Mutational analysis of metastatic lymph nodes from papillary thyroid carcinoma in adult and pediatric patients

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Background. *Limited data are available on the analysis of somatic mutations in metastatic lymph nodes in adult and pediatric patients with papillary thyroid carcinomas.*
Methods. *A total of 92, microdissected, formalin-fixed, paraffin-embedded tissue specimens from 39 patients were analyzed for the presence of somatic mutations utilizing the ThyGenX next-generation sequencing test.*
Results. *Somatic mutations were detected in 67% of papillary thyroid carcinoma specimens. The majority of patients with synchronous and all 6 patients with radioactive iodine–resistant (metachronous) metastatic lymph nodes contained BRAF mutations. Four patients had mutations detected in their metastatic lymph nodes that were not detected in their primary tumors. For the most part, BRAF mutations were seen in adults, and RAS mutations were seen in children.*
Conclusion. *Findings of different mutations in metastatic lymph nodes compared with the primary papillary thyroid carcinomas are probably the result of tumor heterogeneity. The presence of the BRAF mutation in metastatic lymph nodes might be responsible for the recurrence of papillary thyroid carcinomas and resistance to radioactive iodine therapy. The good prognosis observed in papillary thyroid carcinomas found in pediatric and young adult patients might be explained by the predominance of RAS rather than BRAF mutations. (Surgery 2017;161:176-87.)*

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Differentiated thyroid cancer (DTC) comprises the vast majority (>90%) of all thyroid cancers.[1] The yearly incidence of DTC has nearly tripled from 4.9 per 100,000 in 1975 to 14.3 per 100,000 in 2009. The risk of malignancy in adults is reported to occur in 7–15% of thyroid nodules.[2] This change has been attributed to an increase in the incidence of papillary thyroid carcinomas (PTC), with a majority of the cases being micro-PTC (<1 cm). Better detection and early diagnosis by neck imaging studies, such as ultrasonography, probably accounts for this increase in apparent incidence. In spite of the increasing incidence of DTC, the mortality rate remains low because the disease often remains stable for years.[3,4]

Although thyroid nodules are uncommon in children, the risk of malignancy approaches 26%, which is much greater than seen in adults.[1,2,5-7] Children with PTC are more likely to have regional lymph node (LN) involvement and extrathyroid extension. Up to 45% of cases are expected to have pulmonary metastasis at the time of initial presentation.[5] In spite of extensive disease at the initial clinical presentation, children are much less likely to die from disease than the adults.[1,5]

No laboratory or pharmaceutical company had any commercial involvement in this study. Interpace Diagnostics LLC provided complimentary molecular evaluation of the samples. There was no financial support or compensation to the investigators, the patients, or the laboratory itself.

Presented as an oral podium presentation at the 37th annual meeting of the American Association of Endocrine Surgeons in Baltimore, MD, April 10–12, 2016.

Accepted for publication October 6, 2016.

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0039-6060/$ - see front matter

http://dx.doi.org/10.1016/j.surg.2016.10.002

It is expected that even with development of pulmonary metastases, many children will have persistent but stable disease after radioactive iodine (RAI) therapy.[5,8,9]

Evaluation of the mutations seen within thyroid nodules has become one of the newly developed diagnostic tools that helps with earlier detection of thyroid cancer and even determines prognosis and response to therapy. Recently, the guidelines of the American Thyroid Association for adults and children with thyroid nodules have recommended that mutational analysis of thyroid nodules with atypical cytology be carried out to improve the diagnostic assessment and the risk of malignancy.

This analysis also assists in disease management, that is, surveillance versus diagnostic operation.[1,5,10] Clinical tests developed in several laboratories[11,12] utilize different technologies to detect somatic mutations in thyroid nodule DNA. Most commonly, the analysis consists of detecting genetic mutations, such as those present in BRAF, NRAS, KRAS, HRAS, RET/PTC, and PAX8/PPARγ genes.[1] Currently, next-generation sequencing (NGS) is the technology of choice for detecting genetic mutations. Utilizing genome-scale technologies and novel biomarker candidates, such as miRNAs, we are able to identify now distinct histopathologic tumor types at various stages of differentiation.

These miRNAs are small, highly conserved RNA molecules that regulate key cellular processes, such as cell-cycle progression, cell differentiation, proliferation, and survival.[12] The presence of specific mutations in DTC has been shown to correlate with a poor prognosis of thyroid cancer. One of the genetic mutations found associated commonly with the poor prognosis is BRAF V600E, the presence of which independently predicts central neck nodal metastases in PTC and may correlate with RAI resistance and local recurrence.[13-15]

Although mutational analysis of DTC has been available for some time, limited data are available on somatic mutation status in metastatic LNs, in both adult and young adult patients with DTC. Also, the molecular events that lead to the development of LN metastasis and lack of response to RAI therapy in a fraction of DTC patients are also unknown. Studying the mutational profile in DTC in synchronous and metachronous metastatic LNs may help in management and treatment of metastatic disease. Therefore, the goal of this study was to identify associations of various mutations in PTC and mutations in their corresponding synchronous and metachronous metastatic LNs. This might explain the difference in the prognosis of various DTCs, specifically PTC, and might lead to better patient management with consideration of targeted therapies.

With this in mind, we evaluated (1) the mutational profile of synchronous metastatic LNs from PTC and the relationship with the primary (PTC), (2) the profile of synchronous central neck, level 6 LN metastases (CNLN), and the more distant lateral neck LN metastases, (levels 2,3,4, and 5), (3) the mutational profile in PTC of adult versus pediatric and young adult patients which might explain their different presentation and prognosis, (4) the differences in the mutational profile of the DTC and the corresponding metastatic LNs in pediatric and adult patients, and (5) the mutational profiles of metachronous metastatic LNs in patients who developed recurrences after prior thyroidectomy, followed by RAI ablation.

In this report, we sought to provide evidence to support the hypothesis that the presence of the BRAF mutation in metastatic LNs might be a marker for eventual recurrence or metastasis of PTC, and its resistance to RAI therapy.

METHODS

Study design. A total of 92 specimens (43 thyroid tumors, 49 LNs) in 39 patients (including 8 [aged between 12 and 22 years] pediatric and young adult patients) who underwent operative therapy for DTC were utilized for this study. No patient had a history of radiation exposure. All tumor specimens were deidentified and were provided to the pathologist retrospectively for microtome tissue sectioning, and for the repeat assessment of their tumors. Deidentified formalin-fixed paraffin-embedded (FFPE) slides were then sent for genetic mutation analysis. The results were blinded to all the investigators (including the scientists at the molecular laboratory) until all the test results were completely available. Correlations between the mutations seen in the primary, versus nodal synchronous or metatchronous metastasis, were made. Institutional review board approval was obtained for this study.

Patient groups. The patients were divided into 4 groups: (1) total thyroidectomy only (TT); (2) re-exploration with modified radical neck dissection only (MRND) or central neck (levels 6–7) LN dissection (CNLD) or both for those patients who had prior TT, and were treated with RAI and developed recurrence subsequently (metachronous LN metastases) or had persistent disease despite RAI therapy (MRND/CNLD); (3) TT and CNLD for synchronous metastases (TT + CNLD); and (4) TT,

Table I. Patient demographics and tumor characteristics

Groups	*Specimen no.*	*Patient ID*	*Sex*	*Age*	*Pathology*	*Variant*	*Tumor size (cm)/LNs*
Adult groups							
TT	1–2	1	Female	67	PTC	Classic	1.3/3.5
	3	2	Male	29	PTC	Classic	1.5
	4–5	3	Female	47	PTC	Classic	0.1/0.8
	6	4	Female	69	PTC	Follicular	1.2
	7	5	Female	86	PTC	Follicular	1
	8	6	Female	51	PTC	Follicular	1.5
	9	7	Female	70	PTC	Classic	2
	10	8	Female	83	PTC	Follicular	1.1
	11	9	Male	56	PTC	Classic	4.8
	12	10	Female	38	PTC	Follicular	0.5
MRND/CNLD	13–14	11	Male	44	PTC	Classic	LNs
	15–16	12	Female	37	PTC	Classic	LNs
	17	13	Female	70	PTC	Classic	LN
	18	14	Female	51	PTC	Classic	LN
	19	15	Male	70	PTC	Classic	LN
	20	16	Female	62	PTC	Classic	LN
TT + CNLD	21–25	17	Female	41	PTC	Classic	1.4/LNs
	26–28	18	Female	41	PTC	Classic	1.1/LNs
	29–31	19	Male	30	PTC	Classic	4.2/LNs
	32–35	20	Female	28	PTC	Classic	2.5/2.5/LNs
	36–39	21	Female	37	PTC	Classic	0.8/3.8/LNs
	40–43	22	Male	48	PTC	Classic	0.4/1.0/LNs
	44–48	23	Female	33	PTC	Classic	0.7/0.4/4.5/LNs
	49–50	24	Male	64	PTC	Classic	0.9/LN
	51–54	25	Female	38	PTC	Classic	5.0/0.6/LNs
	55–56	26	Female	67	PTC	Classic	0.9/LN
	57–58	27	Female	45	PTC	Classic	0.8/LN
TT + CNLD + MRND	59–66	28	Male	48	PTC	Classic	2.5/1.3/1.6/LNs
	67–72	29	Female	56	PTC	Follicular	1.5/1.0/LNs
	73–77	20	Male	26	PTC	Classic	3.6/LNs
	78–79	31	Female	25	PTC	Classic	1.3/LN
P&YA groups							
TT	80	32	Female	22	PTC	Follicular	2.5
	81	33	Female	18	PTC	Classic	0.6
	82	34	Female	21	PTC	Follicular	0.8
MRND/CNLD	83–84	35	Female	18	PTC	Classic	LN
TT + CNLD	85–86	36	Female	22	PTC	Classic	0.6/LN
	87–88	37	Male	12	PTC	Classic	2/LN
	89–90	38	Female	22	PTC	Classic	1.5/LN
TT + CNLD + MRND	91–92	39	Female	21	PTC	Classic	1.6/LN

P&YA, Pediatric and young adult patients group.

CNLD, and simultaneous MRND for synchronous LN metastases (TT + CNLD + MRND). Pediatric and young adults were also separated into similar groups. RAI-resistant PTC tumors were defined as those that recurred or progressed, despite initial and/or repeated doses of RAI.

Pathology. Archival, FFPE tissue blocks were sectioned serially at a 5-micron thickness, and multiple slides were prepared. A central, hematoxylin-eosin stained, scout section was examined through a light microscope, and the areas containing tumor were outlined with a marking pen. In cases of multifocal PTC tumors, the largest (dominant) thyroid tumor was tested for mutational analysis using the Thy-GenX NGS test; similarly, the largest metastasis LN was selected for mutational analysis.

The areas of tumor marked on the hematoxylin-eosin stained slides were matched to corresponding unstained sections. Areas of tumor were microdissected from the unstained slides for the isolation of total nucleic acids (TNA) using the

Table II. Summary of mutational analysis of adult and pediatric and young adult groups

Total thyroid PTC groups	*Thyroid PTC (adult/P&YA)*	*BRAF (adult/P&YA)*	*KRAS (adult/P&YA)*	*NRAS (adult/P&YA)*	*RET/PTC1 (adult/P&YA)*	*Total thyroid mutations (adult/P&YA)*
TT	15 (12/3)	5 (5/0)	1 (0/1)	0	1 (0/1)	7 (5/2)
TT + CNLD	20 (17/3)	14 (12/2)	0	0	2 (2/0)	16 (14/2)
TT + CNLD + MRND	8 (7/1)	3 (3/0)	1 (0/1)	1 (0/1)	1 (0/1)	6 (5/1)
Total thyroid PTC	43 (36/7)	22 (20/2)	2 (0/2)	1 (0/1)	4 (3/1)	29 (24/5) (67%)
LNs						*Total LN mutations*
Total LN mutations		19 (18/1)	1 (0/1)	1 (0/1)	6 (6/0)	27 (24/3)

P&YA, Pediatric and young adult patients group.

RecoverAll kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. The NGS-based, ThyGenX test was carried out from the isolated TNA for somatic mutation profiling. The ThyGenX assay then interrogated alterations in the relevant genes of thyroid cancer, including 9 hotspot regions in the BRAF, HRAS, KRAS, NRAS, and PIK3CA genes, and 5 fusions in the PAX8/PPARγ and RET/PTC genes.[12]

Sequencing data were analyzed using a proprietary bioinformatics pipeline by comparing the sequencing data to the COSMIC database. DNA mutants were called positive at a threshold of >5% variance. The RNA fusion mutants were called positive when the number of observed sequences of the fusion transcript was greater ($P \leq .05$ by Poisson test) than 10% of the housekeeping TATA-box binding protein transcripts. Percent variance was a measure of the number of mutations as a percentage of the total number from NGS analysis. For example, a 50% mutation variance in BRAF V600E meant that 100,000 out of 200,000 sequenced genes were mutant. Also, a 50% mutation variance could indicate that one copy of the chromosome had been mutated in 100% of the cells.[12]

RESULTS

A total of 92 samples (43 thyroid tumors, 49 LNs) were studied in 39 patients; 80% (31/39 patients) were the classic variant of PTC (Table I). Somatic mutations were detected in 67% (29/43) of PTC. Mutations were seen in 67% (24/36) of adult thyroid tumors and in 71% (5/7) of thyroid tumors from pediatric and young adults (Table II). A majority of the patients with synchronous metastatic LNs (70%), including all 6 patients with RAI-resistant (metachronous) LNs, contained BRAF mutations (Table II).

Mutational analysis in the TT adult group. We analyzed 12 thyroid PTC samples from 10 patients. The tumor sizes ranged from 0.1 to 4.8 cm. (Table III). Three (25%) were micro-PTC, 5 (42%) were follicular variants of PTC, and 7 (58%) were classic variants. Five of the 12 (42%) PTC tumors had BRAF mutations, including one 0.5-cm micro-PTC. Five tumors had no detectable mutations, and 2 were designated as sample failures. The percent variance of BRAF was between 20% and 45% in macro-PTC and 6% in the 0.5-cm micro-PTC (Table III).

Mutational analysis in the MRND/CNLD adult group. Eight samples from LNs in 6 patients with PTC were analyzed (Table IV). All the metastatic LNs contained the classic variant of PTC. Patients presented with metachronous metastatic LNs anywhere from 1–10 years (average 4.3 years) after the initial TT and 1–9 years after the last dose of RAI (average 3.3 years). All received 1 to 2 treatments of RAI with cumulative doses from 74–250 mCi (average dose 206 mCi; Table V). Despite the recurrence, and development of LN metastases that showed resistance to RAI, some of those patients seemed to have stable disease for several years without either progression or regression after RAI therapy (see patient 11 in Table V).

Several patients were followed without operative intervention but, eventually underwent MRND or CNLD. All patients in this group with metachronous metastatic LNs (100%) had a positive mutational profile. The LNs of 5 patients had BRAF mutations (20–30% variance), and 1 had a RET/PTC1 rearrangement. Two of those 5 patients had central neck, level 6 nodal recurrences. Both were BRAF positive. One of those 2 patients had recurrent PTC (level 6 LN) in spite of the initial thyroid tumor being a follicular thyroid carcinoma. A mutational profile for the original tumor was not done.

Table III. Mutational analysis in total thyroidectomy group only (TT)

Specimen no.	*Patient ID*	*Sex*	*Age*	*Pathology*	*Variant*	*Tumor size (cm)*	*Mutation status*
1	1	Female	67	PTC	Classic	1.3	BRAF 40%
2						3.5	BRAF 45%
3	2	Male	29	PTC	Classic	1.5	BRAF 20%
4	3	Female	47	PTC	Classic	0.1	No mutation
5						0.8	No mutation
6	4	Female	69	PTC	Follicular	1.2	No mutation
7	5	Female	86	PTC	Follicular	1	Sample fail
8	6	Female	51	PTC	Follicular	1.5	No mutation
9	7	Female	70	PTC	Classic	2	BRAF 32%
10	8	Female	83	PTC	Follicular	1.1	Sample fail
11	9	Male	56	PTC	Classic	4.8	No mutation
12	10	Female	38	PTC	Follicular	0.5	BRAF 6%

BRAF % indicates percentage of the mutated gene variance; no mutation indicates no mutation detected.

Table IV. Mutational analysis in MRND/CNLD group

Specimen no.	*Patient ID*	*Sex*	*Age*	*Pathology*	*Variant*	*Lymph node*	*Mutation status*
13	11	Male	44	PTC	Classic	LN level 3	BRAF (28%)
14						LN level 5	No mutation
15	12	Female	37	PTC	Classic	LN level 2	No mutation
16						LN level 3	BRAF (28%)
17	13	Female	70	PTC	Classic	LN Right level 2–5	BRAF (25%)
18	14	Female	51	PTC	Classic	LN Right level 2–5	RET/PTC1
19	15	Male	70	PTC	Classic	Thyroid bed LN level 6	BRAF 30%
20	16	Female	62	PTC	Classic	Thyroid bed LN level 6	BRAF 20%

Patients who had prior TT then were treated with RAI and subsequently developed recurrence (metachronous LN metastases) or had persistent LN disease despite RAI therapy. BRAF % indicates percentage of the mutated gene variance.

Table V. RAI-resistant group (the same patients as in Table IV MRND/CNLD)

Patient ID	*Age*	*TT date*	*LN size pre-RAI, cm (date)*	*First RAI mCi (date)*	*LN size pre-second RAI, cm (date)*	*Second RAI mCi (date)*	*LN size post-RAI, cm (date)*	*Time after the last RAI (mo)*	*MRND/CNLD date*
11	44	4/08	1.4 (4/08)	105 (8/08)	1.4 (6/09)	148.5 (9/09)	1.7 (12/11)	31	1/12
12	37	10/08	NA	102 (1/09)	NA	No	1.0 (2/11)	34	8/11
13	70	2/07	NA	103 (2007)	1.3 (4/11)	122 (6/11)	3.5 (6/12) 2.1 (6/13)	24	6/13
14	51	7/12	NA	74 (1/12)	NA	No	1.4 (11/13)	25	2/14
15	70	10/03	NA	122 (1/04)	NA	No	0.9 (7/13)	117	9/13
16	62	1/14	NA	100 (3/14)	NA	No	1.0 (6/15)	13	4/15

LN metastasis size is measured by thyroid/neck ultrasonography. CNLD, level 6; MRND, lateral neck levels 2, 3, 4, and 5.

Mutational analysis in the TT + CNLD adult group. A total of 35 samples from 11 patients (17 thyroid tumors and 18 corresponding synchronous central neck LNs) were analyzed (Table VI). All were the classic variant of PTC. Some had bilateral multifocal tumors (sizes 0.4–4.5 cm) and 9 of 17 were micro-PTC; 71% (12/17) of these PTC thyroid tumors had BRAF mutations. Their variance was 17–41% for the macro-PTC patients, and 6–31% for the micro-PTC patients. Two patients had RET/PTC1 rearrangements (12%). One patient had 2 tumors: one 0.7-cm tumor had a RET/PTC1 mutation and the other 0.4-cm tumor had a BRAF mutation.

Fifty-three percent (9/17) of the LNs also tested positive for mutations; 6 of the 9 showed the BRAF mutations. One of those 6 patients had a multifocal micro-PTC, which showed no mutation in the dominant thyroid tumor, but demonstrated the BRAF mutation in the metastatic LN. Two patients

Table VI. Mutational analysis in the TT + CNLD group

						Tumor size (cm)	*Mutation status*	
Specimen	*Patient ID*	*Sex*	*Age*	*Pathology*	*Variant*	*Thyroid tumor (cm) right/left*	*Thyroid tumor*	*Lymph node*
21–25	17	Female	41	PTC	Classic	1.4 R	BRAF (17%) R	BRAF (29%) R
26–27	18	Female	41	PTC	Classic	1.1 L	BRAF (17%) L	BRAF (7%) L
28								No mutation R
29–30	19	Male	30	PTC	Classic	4.2 L	No mutation L	No mutation L
31							NA	No mutation R
32–34	20	Female	28	PTC	Classic	2.5, 2.5 (R, R)	BRAF (38% & 41%) (R&R)	BRAF (24%) R
35						No tumor L		BRAF (7%) L
36–37	21	Female	37	PTC	Classic	0.8 R	BRAF (17%)	No mutation
38–39						3.8 L	BRAF (27%)	No mutation
40–41	22	Male	48	PTC	Classic	0.4 R	BRAF (11%) R	BRAF (13%) R
42–43						1.0 L	BRAF (6%) L	Sample failure L
44–46	23	Female	33	PTC	Classic	0.7, 0.4 (R, R)	RET/PTC1 & BRAF (7%)	RET/PTC1 R
47–48						4.5 L	RET/PTC1 L	RET/PTC1 L
49–50	24	Male	64	PTC	Classic	0.9 L	No mutation L	BRAF (15%) L
51–52	25	Female	38	PTC	Classic	5.0 R	BRA (27%) R	No mutation R
53–54						0.6 L	No mutation L	No mutation L
55–56	26	Female	67	PTC	Classic	0.9 R	BRAF (31%) R	No mutation R
57–58	27	Female	45	PTC	Classic	0.8 (pyramidal lobe)	BRAF (9%)	RET/PTC1 (pretracheal LN)

BRAF and KRAS % indicates percentage of the mutated gene variance.
No mutation, No mutation detected; *L*, left; *R*, right; *NA*, not applicable.

had RET/PTC1 rearrangements in metastatic LNs. Another patient had RET/PTC1 rearrangement within the metastatic LN, and a BRAF mutation in the original 0.9 cm PTC. These 2 patients had findings of different mutations in metastatic LNs (BRAF and RET/PTC1) that were not detected in the tumor of origin.

Mutational analysis in the TT + CNLD + MRND adult group. A total of 21 samples from 4 patients with PTC were analyzed. Of 8 thyroid tumors sizes 1.0–3.6 cm, some were multifocal PTC, 6 were synchronous central neck LNs, and 7 were lateral neck LNs (Table VII). All 4 patients had mutations detected in either the thyroid tumors, LNs, or both: 2 patients (3 tumors, classic variant of PTC) had BRAF in the thyroid (variance 20–39%), one had NRAS (a 1.5-cm follicular variant of PTC, variance 16%), one had RET/PTC1 (classic variant of PTC).

Lateral neck LNs (MRND) in 3 of 4 patients had positive mutation analyses: 2 with BRAF (16% and 26% variance) and one with RET/PTC1. One patient with no mutation in the thyroid tumor on the right side had an NRAS (16% variance) mutation on the left side of the thyroid, and a BRAF mutation (17% variance) in the central level 6 LN on the right, but no mutation in the lateral neck. The preoperative workup included a mutational profile of the thyroid tumor and tested positive for the BRAF mutation. In the current study, we had a sample failure of the test on the thyroid tumor.

Patients with lateral neck LN mutations always had corresponding central neck LN mutations as well. One patient with RET/PTC1 rearrangement in the 3.6-cm PTC had a BRAF (29% variance) mutation in the corresponding left central neck level 6 LN. We did not see a BRAF mutation in the thyroid tumor. The same patient also had RET/PTC1 rearrangements in a central (level 6), 2 lateral level 2 LNs, and one level 3 LN on the same side.

Mutational analysis in the pediatric and young adult groups. A total of 13 samples (7 thyroid tumors, and 6 LNs) in 8 patients with DTC (all PTC, 2 follicular variants, 6 classic variants) were characterized (Table VIII). Three patients were in the TT group, 1 was in the MRND/CNLD group, 3 were in the TT + CNLDgroup, and 1 was in the TT + CNLD + MRND group. None of these patients had a history of radiation exposure. Five had positive thyroid genetic alterations: 1 RET/PTC1, 2 KRAS, and 2 BRAF. Two of 3 patients in the TT group had mutations in their thyroid

Table VII. Mutational analysis in the TT + CNLD + MRND group

Specimen ID	*Patient ID*	*Sex*	*Age*	*Thyroid tumor size (cm)*		*Mutation analysis*					
						Right			*Left*		
				Right	*Left*	*Thyroid*	*Central LN*	*Lateral LN*	*Thyroid*	*Central LN*	*Lateral LN*
59–66	28	Male	48	2.5 and 1.3	1.6	BRAF (20%)	BRAF (27%) and BRAF (29%)	No mutation	BRAF (39%)	BRAF (37%)	BRAF (33%)
67–72	29	Female	56	1	1.5	sample failure (*)	BRAF (17%)	No mutation (levels 3 and 5)	NRAS 16%	Not done	Not done
73–77	30	Male	26	None	3.6	NA	NA	NA	RET/PTC1	RET/PTC1 and BRAF (29%)	RET/PTC1 (level 2) and RET/PTC1 (level 3)
78–79	31	Female	25	None	1.3	NA	NA	NA	BRAF (35%)	Not done	BRAF (16%)

*Preoperative FNA biopsy showed BRAF mutation.
BRAF and NRAS % indicates percentage of the mutated gene variance.
No mutation, No mutation detected; *None*, no tumor.

tumors: one had a 2.5-cm PTC with a RET/PTC1 rearrangement, and another had a 0.8-cm PTC with a KRAS (42% variance) mutation. One patient with 0.6-cm PTC had no mutation.

One patient in the MRND/CNLD group had no mutation in the level 2 metachronous metastatic LNs. Two of 3 patients in the TT + CNLD group had a BRAF mutation in the thyroid (24% and 34% variance). Of 2 patients with a BRAF mutation in the thyroid tumor, one had no synchronous mutations in the central neck LNs and another had an LN with a BRAF (34% variance) mutation. The third patient with no mutation in the thyroid tumor had an NRAS (39% variance) mutation in a corresponding level 6 LN. One patient with a 1.6-cm PTC in the TT + CNLD + MRND group had a KRAS (25% variance) mutation in the thyroid tumor and no mutations in the synchronous lateral neck LNs.

Mutations detected in the LNs but not in the primary tumor. We found 4 patients with LN mutations in synchronous corresponding metastatic LNs that were not present in the primary thyroid tumors. Two were with BRAF, one was with RET/PTC1, and one was with KRAS seen in the pediatric and young adult group (Table IX). Two of those patients with BRAF-positive LNs had multiple metastatic LNs. Patient 24 had a total of 9 metastatic LNs, and patient 30 had 17 metastatic LNs.

DISCUSSION

Our analysis of combined adult and pediatric and young adult groups showed that 67% of PTCs harbored mutations. We noted 67% in the adult and 71% in the pediatric and young adult groups. The BRAF mutation was present in 43% of all PTCs (46% in adult and 29% in pediatric and young adult patients). In addition, we found one NRAS mutation in an adult, 2 KRAS mutations in the pediatric and young adult group, and 4 RET/PTC1 rearrangements in thyroid tumors: 3 in adults and 1 in the pediatric and young adult group (Table II).

We found total of 27 mutations in corresponding (synchronous) and metachronous metastatic LNs. Seventy percent of the LNs carried the BRAF mutation (75% in adult), and 22% carried a RET/PTC1 rearrangement. We also found one LN with a KRAS mutation and one with NRAS mutations. The majority of the metastatic LNs were the classic variant of PTC, especially those that were BRAF positive.

Central neck LN metastases were common in PTC and developed in up to 35% of the patients.[1,16] The latency period between the metastatic event

Table VIII. Mutational analysis in pediatric and young adult patients (P&YA)

Group	*Specimen ID*	*Patient ID*	*Sex*	*Age*	*Thyroid tumor (cm)*	*Variant*	*Right*	*Left*
TT (n = 3)	80	32	Female	22	PTC 2.5	Follicular	RET/PTC1	NA
	81	33	Female	18	PTC 0.6	Classic	None	NA
	82	34	Female	21	PTC 0.8, 0.5,0.2	Follicular	KRAS (42%)	NA
MRND/CNLD (n = 1)	83–84	35	Female	18	Pathology	Classic	LNs	Mutation
					PTC		LN L 2/LN L 5	No mutations
TT + CNLD (n = 3)					Thyroid tumor (cm)		Right thyroid/ right CNLN	Left thyroid/left CNLN
	85–86	36	Female	22	None/PTC (0.6)	Classic	NA	No mutation/NRAS (39%)
	87–88	37	Male	12	PTC (2.0)/none	Classic	BRAF (24%)/ no mutation	NA
	89–90	38	Female	22	None/PTC (1.5)	Classic	NA	BRAF (24%)/BRAF (34%)
TT + CNLD + MRND	91–92	39	Female	21	Thyroid tumor (cm)	Classic	Right thyroid/ central LN/lateral	Left thyroid/central LN/lateral LN
					PTC 1.6/none		KRAS (25%)/not done/ no mutation	NA

BRAF, KRAS, and NRAS % indicates percentage of the mutated gene variance. CNLD, level 6; MRND, lateral neck levels 2, 3, 4, and 5.
No mutation, No mutation detected; *None*, no tumor.

Table IX. Summary of samples with mutation detected in the LNs and not the primary tumor

Patient ID	*Age*	*Group*	*Thyroid tumor mutations*	*LN mutations**
24	64	TT + CNLD	None	BRAF
27	45	TT + CNLD	BRAF	RET/PTC1
30	26	TT + CNLD + MRND	RET/PTC1	BRAF
36	22	P&YA TT + CNLD	None	NRAS

*Mutation detected in the LNs and not the primary tumor.
CNLD, level 6; MRND, lateral neck levels 2, 3, 4, and 5.
P&YA, Pediatric and young adult patients.

and the subsequent progression of the disease seemed to be regulated by the cancer cells, but may depend on other factors as well. The late progression of the disease is probably not related to the development of new secondary metastases, but rather attributed to changes in the metastatic cells that lie dormant in metastatic locations.[17]

In the current study, we found 4 patients with mutations in synchronous LNs that were not present in the thyroid tumor (Table IX). Two adult patients had BRAF mutations. One patient had no mutations in the dominant thyroid tumor, and another one had a thyroid tumor positive for a RET/PTC1 rearrangement. One patient had a RET/PTC1 rearrangement, and one in the pediatric and young adult group had a KRAS mutation. One patient had a mutation noted on a preoperative fine needle aspiration (FNA), which was not detected in the operative specimen of the primary thyroid tumor. One patient with a multifocal micro-PTC had no mutation in the dominant thyroid tumor, but had a mutation in the corresponding metastatic LN. Two of those patients with BRAF-positive LNs had multiple metastatic LNs: one patient had 9 metastatic LNs, and another patient had 17 metastatic LNs.

The findings of different mutations in the metastatic LNs that were not detected in the tumor of origin may be explained either by the presence of tumor multifocality, heterogeneity, or possibly, the development of new mutations. We have not had the ability to study all multiple, small, micro-PTC cases, and thus it is possible that instead of a dominant PTC tumor, some of those microtumors not tested may have had a BRAF mutation that resulted in a BRAF-positive LN metastasis.

Discordance in mutational analysis results between the FNA biopsy and resected specimens may be the result of tumor heterogeneity. Multiple subclones of tumor cells possessing different mutations may be present within a single tumor. Due to limited sampling, FNA biopsies may not contain all the tumor cell subclones. Microdissection of histologic sections has the potential to provide a greater, more representative sample of tumor cells for mutational analysis despite tumor heterogeneity.[16] Some reports have described the development of de novo BRAF mutations in metastatic LNs that could be attributed to loss of dormancy and accelerated progression of metastatic PTC.[18,19]

We have presented previously a small feasibility study on the molecular analysis of metastatic LNs by preoperative, ultrasonography-guided, FNA biopsy of the thyroid and corresponding LNs in patients with DTC. These are cases in which we detected mutations in the LNs that were not present in the primary tumor.[20] Long-term consequences of a different mutational profile within the metastatic LNs compared with the tumor of origin have not been studied, and greater durations of follow-up are needed to determine if these differences in the mutational profile are associated with a worse prognosis.

In this study, we found that all the patients with metachronous metastatic LNs in RAI-resistant PTC had a positive mutational profile. All PTC metastatic LNs that we studied in the MRND/CNLD group carried mutations; BRAF mutations were seen in 5 patients, and the RET/PTC1 rearrangement was seen in one patient. We hypothesize that the presence of BRAF (or RET/PTC1) mutations in metastatic LNs a number of years after the thyroidectomy followed by a high dose of RAI therapy may confer resistance to RAI therapy.

Despite the recurrence and development of LN metastases resistant to RAI, some of those patients appeared to have stable disease for several years without progression or regression of the disease after additional RAI therapy. Based on some of our data and evaluating the size of the nodules before and after the RAI therapy (Table V), we speculate that BRAF mutations might not be the only factor that influences disease progression, and that additional factors could be involved. For example, some reports have suggested that mutations in the TERT promoter region could also be responsible for the aggressive behavior of the thyroid tumors.[10,21] Unlike BRAF mutations, RAS mutations were not found on post-RAI analyses of metastatic LNs in our small group of patients. It has yet to be determined if RAS-positive PTC and RAS-positive metastatic LNs respond better to RAI therapy. More studies are needed to answer

this question. The knowledge of specific LN mutations in RAI-resistant DTC may have clinical utility for patient management and may help in the choice of therapy.

In analyzing our data with PTC and central or lateral neck LNs, we found similar BRAF mutations in corresponding synchronous metastatic LNs in several adult patients. This finding may explain the presence of more aggressive disease and resistance to RAI therapy in adult patients. Other factors are probably also involved in the aggressiveness of the tumor, because not all of those patients showed a rapid progression of the disease, but rather had a very indolent course. We also found that multifocal PTCs contain different mutations in different foci within the same thyroid gland. As seen in some of our patients, mutations in the primary tumor are often different from those seen in the nodal metastases.

The underlying process that led to a better prognosis in the pediatric and young adult group, in spite of a more extensive disease at presentation, remains unknown. In our series and in contrast to adult patients, the pediatric and young adult group had a more favorable course of their disease despite being more likely to have regional LN involvement and extrathyroid extension; up to 45% of this group will have pulmonary metastasis at the initial presentation.[5,22] Despite extensive disease at the initial clinical presentation and in contrast to adults, children are much less likely to die from the disease (<2%).[1,5] Even with the development of pulmonary metastases, many children will have persistent but stable disease after RAI therapy.[5]

Limited data are available on the molecular profile of pediatric DTC and specifically PTC. Several prior studies of pediatric PTC have reported prevalence of RET/PTC rearrangements in the pediatric population, primarily from radiation exposure (Chernobyl catastrophe).[5,22,23] Some studies have shown the presence of the BRAF mutation in pediatric thyroid carcinomas, but others have not.[24] One study showed that the BRAF mutation is rare and almost absent in children with PTC.[5,25] A different spectrum of mutations may explain different or better response to RAI therapy and the low the mortality in children with PTC.[5,8,9,24]

Finally, in our series, analysis of somatic mutations in microdissected, FFPE tissue from metastatic LNs and from corresponding primary thyroid foci of PTC in patients in the pediatric and young adult group showed that 5 of 8 patients had positive mutational analysis in the primary focus (1 RET/PTC1, 2 KRAS, 2 BRAF), and one patient had an NRAS mutation detected in a metastatic LN but not in the primary tumor (Tables VIII and IX).

Even with the small number of this patient group, there was a predominance of RAS (KRAS and NRAS) mutations compared with the adult group. Two pediatric and young adults had KRAS mutations in the primary focus and one had an NRAS mutation in a corresponding metastatic LN but not in the primary focus. We can speculate that better prognosis of PTC in the pediatric and young adult group might be due to the predominance of RAS rather than BRAF mutations. Greater durations of follow-up and more studies are needed to prove this point.

In our study, we also reported a percentage of specific mutations (variance) in each BRAF-, KRAS-, or NRAS-positive tumor or metastatic LN. A high percentage of BRAF (V600E) alleles (≥30%) defined the presence of PTC molecular subtypes and has been reported as predictive of a poorer disease outcome.[26] We noted a similar trend in our study. The percentage of BRAF variants was slightly less in micro-PTC and greater in macro-PTC, but more studies and greater follow-up are needed to find the statistical significance of these findings.

In conclusion, although the study is limited in the number of the patients in each group, we observed the following trends: PTC in the pediatric and young adult group seems to have a different mutation profile than in adult patients with PTC. Pediatric and young adult patients have less BRAF and more RAS mutations in the tumors. All PTC metachronous metastatic LNs that were RAI-resistant carry the BRAF mutation, even several years after the RAI treatment. Most of the synchronous, lateral neck LN metastases carry mutation markers that are similar to the original thyroid tumor.

Nevertheless, we have found mutations that developed in the corresponding metastatic LNs but were not present in the original thyroid tumor. The long-term consequences of this phenomenon have not been studied and greater follow-up is needed to determine if they cause changing of the tumor profile and worsening of the prognosis of the cancer. It is expected that the assessment of the mutational profile of metastatic LNs from DTC (and PTC specifically) will help in management of recurrent and RAI-resistant disease by utilizing other modalities, such as personalized, target-directed therapy with tyrosine kinase inhibitors that could be matched to the specifically detected mutation.

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DISCUSSION

Dr Jennifer Rosen (Washington, DC): I am particularly interested in hearing more about this group of patients with a delayed modified radical neck dissection. In particular, we have a sort of pesky group of patients now where perhaps they have had their neck dissection, had radioactive iodine, had dosimetry, and we are now observing these 5-, 6-mm nodes still remaining in the lateral neck. I am wondering if you could comment on how we might be able to use this in deciding whether those patients should go for redo modified radical neck dissection, another attempt at

dosimetry, or maybe even alcohol ablation or some other treatment for them.

Dr Alexander L. Shifrin: I think we can use molecular profile. At the end, when we know that we have limit with the dosimetry, we cannot give more radioactive iodine, maybe we can use tyrosine kinase inhibitors if it is not resectable. If it is resectable, I was kind of forced to do surgeries on some patients who come to me with the diagnosis of cancer and insisted on doing surgery rather than radioactive iodine therapy.

For example, I had one patient who was presented to me with a very delayed follow-up (for social reasons), and he had known diagnosis of the papillary thyroid carcinoma a number of years prior. At that time he was found to have metastatic lymph nodes to the lateral neck. Those lymph nodes were stable in size for a number of years. Eventually he modified radical neck dissection. I think that mutational profile would be helpful in answering questions on how to treat metastatic disease and make a decision on either to use radioactive iodine or approach it with target directed therapy by using tyrosine kinase inhibitors.

Dr Jennifer Rosen (Washington, DC): We often find ourselves saying the original tumor was BRAF negative. What is the likelihood that the lymph nodes are going to contain a BRAF mutation? We end up doing a short course of watchful waiting, 3 months or 6 months, or whatever it is, before we decide to commit to re-exploration. So to see that some of your patients, even on recurrence, though the primary was negative, the recurrence was positive was very helpful. Thank you.

Dr Emad Kandil (New Orleans, LA): I liked your paper, but I am struggling to try to make some clinical sense out of this. Can you help me here? Does this mean if a patient had multifocal disease in the thyroid and you had like a big nodule, and it is wild type, it is not positive for BRAF mutation; does this mean that for at least academic reasons you should test the other nodules? We know that with multifocal disease, you have the agreed with surgery and underwent possibility of one that is a BRAF mutant and the other one is not. But since we are talking about the possibility of de novo lymph nodes later on, does this make sense to you? What is the message here?

Dr Alexander L. Shifrin: That is a good question answering your question, I can say that the reason to have different mutations in lymph nodes compare to the primary tumor is either tumor heterogenicity the development of de novo mutation.

Dr Emad Kandil (New Orleans, LA): Well, there is no heterogenicity in the tumor. It is very well established. The tumor itself, once you have a tumor that is a BRAF mutant, it is homogeneous in the single tumor. But if you have multifocal disease, you can have one that is a BRAF mutant and the other one is not. This is very well documented. The question is here, if you have a thyroid mass that is BRAF, it is not mutant, then would you examine the other multifocal ones for BRAF mutations to know if there is maybe a chance for recurrence?

Dr Alexander L. Shifrin: That is right. You may have different mutations in different tumors. I think it is difficult to do a mutational profile of every single small tumor foci in a non-academic institution. This could be good at the NIH probably where they have money to run all the samples. But if you start doing biopsies or studying the mutational profile of every single microPTC, it would be difficult study to complete in our institution. I had patients who had 15 microPTCs within one lobe. It would be difficult to run mutational profile on all of them.

Dr Rasa Zarnegar (New York, NY): I liked your paper. I do have the same concerns that I think when you throw the term "de novo" out, you are assuming that all the tumors you have are homogeneous. Unless you confirm that all the different foci in the original thyroid are the same, to throw out de novo is a very controversial concept. The other question I had was, you also mentioned radioactive iodine resistance. Were these tumors before you did the mutation analysis, was the radioactive iodine resistance confirmed by imaging? And what were the thyroglobulin levels prior to resection of these tumors?

Dr Alexander L. Shifrin: I agree with you on the first part completely. We do not know if it is a de novo mutation until we test all the small foci of PTC within the thyroid specimen. Unfortunately, we had no ability to do so with multifocal disease, and the best effort was made by the pathologist to find the dominant tumor.

With the second question, regarding radioactive iodine. The resistance was defined as persistent disease despite a high dose of radioactive iodine therapy or an increase in the size of the tumor with elevated thyroglobulin level with positive uptake scan.

Those patients who have had RAI therapy and subsequently had no radioactive iodine uptake but had persistent disease and increase in size of the metastatic LNs were also considered as radioactive iodine resistant. In particular, we had patients had multiple lymph nodes metastases despite the RAI therapy and their metastatic LNs have increase in size of metastatic LNs (3 out of 4 patients in MRND group).