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Absence of *BRAF*, *NRAS*, *KRAS*, *HRAS* Mutations, and *RET/PTC* Gene Rearrangements Distinguishes Dominant Nodules in Hashimoto Thyroiditis from Papillary Thyroid Carcinomas

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Abstract Dominant nodules within Hashimoto thyroiditis (HT) may present with unique morphological features that overlap with but are not diagnostic of papillary thyroid carcinoma (PTC). Activating *BRAF* point mutations, *RAS* aberrations, and *RET* rearrangements are mutually exclusive events in the oncogenesis of papillary thyroid carcinoma, and *RET* rearrangements have been previously described in dominant nodules of HT. We identified 28 cases of Hashimoto thyroiditis with a dominant nodule, from 345 consecutive HT thyroidectomies. Screening for *BRAF*, *RET*, *KRAS*, *NRAS*, and *HRAS* mutations, as well as *RET-PTC1* and *RET-PTC3* rearrangements, was performed on paraffin-embedded material from 17 of these dominant nodules. Patients ranged in age from 29 to 76 years and were predominantly female, and the nodules ranged from 1.5 to 6.2 cm. No *BRAF* or *RAS* mutations or RET-PTC rearrangements were identified in a dominant nodule, including those with atypical, worrisome histopathologic features. Of ten cases with diagnostic concomitant or incidental papillary carcinoma, three had a V600E point mutation in *BRAF*, and one case had a *BRAF* exon 15 deletion (600–604E), while the dominant nodules were negative for mutation, supporting the notion that dominant nodules are neither malignant nor precursor lesions, and strict histological, clinical, and molecular criteria must be met for the diagnosis of papillary thyroid carcinoma.

Keywords Hashimoto thyroiditis · Papillary thyroid carcinoma · *BRAF* · *NRAS* · *KRAS* · *HRAS* · RET-PTC · Dominant nodule

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Introduction

Hashimoto thyroiditis (HT) may present with variable histomorphology, including HT with classic features of chronic autoimmune thyroiditis, HT associated with hyperplastic/adenomatous lesions, and HT associated with unequivocal thyroid carcinomas. Some patients with HT, during routine monitoring, have or develop nodules. When these nodules are large (>1 cm) or few in number (so-called dominant nodules), they can be worrisome, prompting fine-needle aspiration biopsy (FNAB). Microscopically, some of these nodules demonstrate certain morphological features suggestive of papillary thyroid carcinoma (PTC) [13, 21].

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These features include nuclear membrane irregularities, clearing, and variable enlargement. Differentiating the morphologic atypia associated with reactive changes of Hashimoto thyroiditis versus true papillary carcinoma can be quite challenging.

It is often the case that lesions that are difficult to classify by FNAB cytology are difficult to classify following excision. This is especially true for well-circumscribed follicular lesions with nuclear membrane irregularities and crowding, especially in the context of Hashimoto thyroiditis, which generally shows oncocytic (Hürthle cell) changes and endocrine atypia. A number of studies have retrospectively investigated the incidence of concomitant carcinomas associated with HT with variable associations [7, 12, 14, 17, 19, 22, 29]. These studies are influenced by whether the concomitant carcinoma was discovered incidentally in a gland resected for known cancer or whether cancer was discovered incidentally in HT following thyroidectomy for some other reason. In the latter circumstance, it is certain that there is some variability in discovery of incidental lesions due to tissue sampling.

The RET/PTC–RAS–BRAF pathway has been investigated in order to understand the relationship between papillary carcinomas of thyroid and oncocytic (Hürthle) changes seen in Hashimoto thyroiditis [1, 8–10, 25]. This has been particularly important for oncocytic changes with varying degrees of nuclear atypia and morphologic features suggestive of papillary carcinoma in dominant nodules of Hashimoto thyroiditis. In this study, we examined 345 consecutive thyroidectomies from patients with a diagnosis of chronic lymphocytic (Hashimoto) thyroiditis. Twenty-eight cases with a dominant nodule were identified, with and without concomitant PTC, of which 17 cases were examined for mutations in *BRAF*, *KRAS*, *NRAS*, *HRAS*, and for *RET/PTC1* and *RET/PTC3* rearrangements.

Materials and Methods

Case Selection

All tissue samples evaluated in this study were obtained from the files of the Brigham and Women's Hospital, Department of Pathology, and were handled in accordance with institutional review board regulations (BWH IRB Protocol 2004P000544). We reviewed 345 consecutive cases of thyroidectomy (partial or complete) with a clinical diagnosis of Hashimoto thyroiditis confirmed by pathology [hematoxylin and eosin (H&E) sections reviewed by P. S. and V. N.] with 28 cases showing a dominant nodule (8.1%). Of these cases, 17 cases (4.9%) were amenable to microdissection for mutational analysis, and all these same 17 cases were suitable for flourescence in situ hybridization (FISH) analysis. After the initial round of *BRAF* analysis, the 17 cases were reexamined blindly and the results found to be concurrent. At this point, dominant nodules (DN) were reassessed for *BRAF*, *KRAS* (exons 1 and 2), *NRAS* (exons 1 and 2), *HRAS* (exons 1 and 2), *RET-PTC1* and *RET-PTC 3*.

Of the 17 dominant nodules analyzed for mutations (Table 2), ten cases (35.7%) had a concomitant carcinoma; nine were PTCs (32.1%), and one was a minimally invasive follicular carcinoma (FC; 3.6%).

BRAF, *KRAS*, *NRAS*, and *HRAS* Mutational Analysis

Mutational analysis of *BRAF* exons 11 and 15 was performed as previously reported [27]. In addition, mutational analyses for exons 1 and 2 of *KRAS*, *NRAS*, and *HRAS* were also performed as previously described [27, 32]. In brief, 5 µm unstained paraffin sections were prepared from blocks containing lesions of interest (either dominant nodule or concomitant carcinoma) by comparison with H&E slides. These lesions were carefully scraped from the glass slides into microfuge tubes with disposable scalpel blades. Specimens were then deparaffinized by serial extractions with xylenes and ethanol and dried at room temperature. The Qiagen mini kit was used to extract DNA (Qiagen, Valencia, CA).

Polymerase chain reaction (PCR) amplification was utilized using High Fidelity PCR system (Roche, Indianapolis, IN). Primer pairs used for *BRAF* amplification [27], *KRAS* amplification [26, 32], *HRAS* amplification [32], and *NRAS* amplification [27, 32] are shown in Table 1.

PCR amplification of genomic DNA was performed using 500 ng of DNA.

Negative controls and water-only templates were utilized in each reaction to exclude contamination of reagents including primers, buffers, and enzymes. Positive controls for mutations were also used as previously reported [4, 20].

Mutation screening was also performed as previously reported [3, 4, 27] using denaturing high-performance liquid chromatography (D-HPLC). Aliquot of 40 µL from the initial PCR products were screened for mutation on a Transgenomic WAVE HPLC system (Transgenomic, Inc., Omaha, NE).

All D-HPLC-detected mutants were bidirectionally sequenced on an ABI 310 sequencer using the Big Dye Terminator kit (Applied Biosystems, Inc., Foster City, CA). One novel mutation was confirmed by three methods: (1) reamplification of the exon and repeat D-HPLC analysis; (2) reextraction of DNA from the tumor tissue, reamplification, and repeat D-HPLC analysis; and (3) bidirectional sequence analysis after each analysis by D-HPLC.

Table 1 Primer pairs for mutational analysis

Gene	Exon	Primer sequence	Orientation
BRAF	Exon 11	5′-TCTGTTTGGCTTGACTTGACTT-3′	Sense
		5′-CGAACAGTGAATATTTCCTTTGAT-3′	Antisense
BRAF	Exon 15	5′-TGCTTGCTCTGATAGGAAAATG-3′	Sense
		5′-AGCATCTCAGGGCCAAAAAT-3′	Antisense
KRAS	Exon 1	5′-TTAACCTTATGTGTGACATGTTCTAA-3′	Sense
		5′-TCATGAAAATGGTCAGAGAAACC-3′	Antisense
KRAS	Exon 2	5′-TTTTTGAAGTAAAAGGTGCACTG-3′	Sense
		5′-TGCATGGCATTAGCAAAGAC-3′	Antisense
HRAS	Exon 1	5′-GGCAGGAGACCCTGTAGGAG-3′	Sense
		5′-AGCCCTATCCTGGCTGTG-3′	Antisense
HRAS	Exon 2	5′-GTCCTCCTGCAGGATTCCTA-3′	Sense
		5′-ATGGCAAACACACACAGGAA-3′	Antisense
NRAS	Exon 1	5′-CACTAGGGTTTCATTTCCATTG-3′	Sense
		5′-TCCTTTAATACAGAATATGGGTAAAGA-3′	Antisense
NRAS	Exon 2	5′-TTGCATTCCCTGTGGTTTTT-3′	Sense
		5′-CCATAATAAAAAGCTCTATCTTCCC-3′	Antisense

RET Rearrangement Analysis by FISH

A detailed description of the methods for *RET* rearrangement analysis has been previously published [27]. Four-micrometer unstained paraffin sections were prepared from blocks containing lesions of interest (either dominant nodule or concomitant carcinoma by comparison with H&E slides). "Break-apart" FISH probes to evaluate *RET* rearrangement were created from bacterial artificial chromosomes (BAC) containing genomic insertions on either side of *RET*. BAC clones were obtained from Children's Hospital Oakland Research Institute (Oakland, CA) and Research Genetics (Huntsville, AL). Two overlapping BACs (RP11-477E7 and RP11-360G12) on the centromeric side of *RET* were labeled with digoxigenin, and two overlapping BACs (RP11-517P14 and RP11-1080O9) on the telomeric side of *RET* were labeled with biotin. To identify the *H4-RET* fusion oncogene, a fusion probe, dual-color FISH approach was used, with probes flanking the *RET* and H4 gene loci. BAC DNA isolations and labeling were performed as previously described [18]. Probe detection was performed using FITC-anti-digoxigenin (1:500) and Alexa Fluor 594-streptavidin (1:500; Invitrogen, Eugene, OR) incubated for 30 min. If fewer than 5% of cells demonstrated splitting apart of the flanking FISH probes, a specimen was deemed negative for rearrangement.

RET Rearrangement Analysis by RT-PCR

RET-PTC1 and RET-PTC3 translocation products were detected in a multiplexed two-color RT-PCR assay with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal positive control. PTC1- and PTC3-specific forward PCR primers were employed with a common RET reverse primer and FAM-labeled Taqman probe, while GAPDH was detected with a Texas Red-labeled probe. The primer and probe sequences included H4 (PTC1) forward primer AAAGCCAGCGTGACCATC, ELE1 (PTC3) forward primer TGGCTTACCCAAA AGCAGAC, RET reverse primer GTTGCCTTG ACCACTTTTC, RET probe 5′FAM-CCAAAGTG GGAATTCCCTCGGA-3′IABlkFQ, GAPDH forward primer AGCCGCATCTTCTTTTGC, GAPDH reverse primer GCCCAATACGACCAAATCC, and GAPDH probe 5′-/5TexRd-XN/TGG GGA AGG TGA AGG TCG GA/3IAbRQSp/-3′. PCR reactions were performed in a Roche LightCycler 480 instrument using a 20-ul reaction volume, with LightCycler 480 Probes Master reaction mix (Roche) and cDNA template derived from 40–200 ng total RNA. The GAPDH probe was used at 0.15 uM, and all other primers and probes were used at a final concentration of 0.3 uM each. Cycling conditions included an initial 10 min denaturing step at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. Samples that scored positive for the RET-PTC1 and RET-PTC3 multiplex were re-tested with each primer pair individually to determine the RET fusion partner.

Results

Clinical Findings and Histology

Of the 345 consecutive cases of HT screened, 28 of these cases had a DN. Of these cases, 17 were studied in greater detail due to availability of sufficient material (Table 2).

Table 2 Mutational analysis in dominant nodules of Hashimoto Thyroiditis

Case	Age (years)	Sex	Diagnosis			Size of DN (cm)	DN	PTC
1	46	F	DN	HT	PTC	1.6		BRAF V600E (2 lesions)
2	56	F	DN	HT	PTC	2.9		BRAF V600E
3	49	F	DN	HT		1.5	WT	
4	76	M	DN	HT		2.1	WT/NR	
5	38	F	DN	HT		2.2	WT	
6	40	F	DN	HT	FC	1.8	WT/NR	
7	61	F	DN	HT	PTC	1.6	WT	BRAF del VKWRV600-604E
8	53	F	DN	HT	PTC	1.5		WT
9	57	F	DN	HT	PTC	6.2	WT	WT
10	35	F	DN	HT	PTC	2		WT
11	29	F	DN	HT	PTC	2.3		WT
12	56	F	DN	HT	PTC	1.5		WT
13	41	F	DN	HT		2	WT	
14	40	F	DN	HT		3.5	WT/NR	
15	45	F	DN	HT	PTC	1.8		WT
16	42	F	DN	HT		2	WT	
17	65	F	DN	HT		2.4	WT/NR	
18–28		NR						

DN Dominant nodule, *HT* Hashimoto thyroiditis, *PTC* papillary thyroid carcinoma, *FC* follicular carcinoma, *WT* wild-type exon 15, *NR* not rearranged

There were 16 females and one male. Females ranged in age from 29–65 years (mean of 47 years; median, 45.5 years). The one male was 76 years old. Of the 16 women with dominant nodules studied, ten (62.5%) had thyroid carcinoma, nine papillary carcinomas, and one follicular carcinoma. The male was cancer-free. Sizes of dominant nodules ranged from 1.5 to 6.2 cm.

Papillary carcinomas identified in association with dominant nodules were classical [4], oncocytic variant [2], and follicular variant [3]. An example of one such concomitant oncocytic variant of papillary carcinoma is shown in Fig. 1a (case 3, Table 2). This lesion shows prominent oxyphilic changes of the cytoplasm with enlarged, cleared-out nuclei with prominent but focal

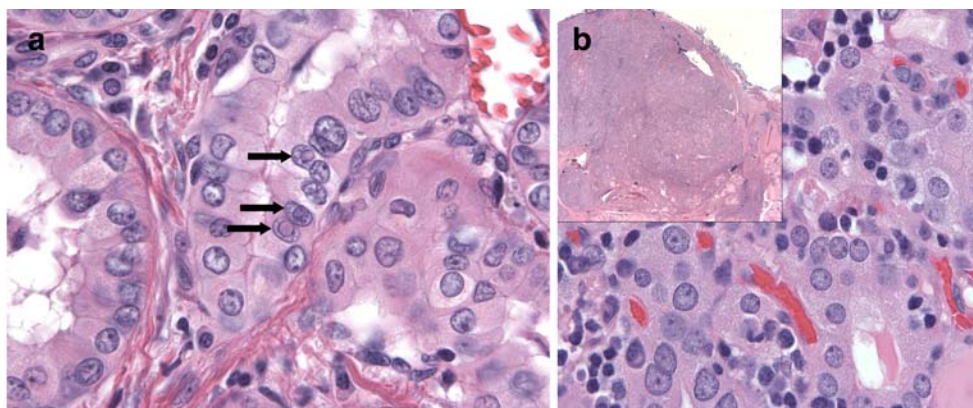

Fig. 1 Papillary thyroid carcinoma (oncocytic variant) adjacent to a dominant nodule in Hashimoto thyroiditis (HT) showing intranuclear pseudoinclusions (*arrows*), membrane irregularity, and grooves (**a**); circumscribed, dominant nodule of HT with lymphocytic infiltration in a background of chronic lymphocytic (Hashimoto) thyroiditis at low power, H&E (*inset*) and high power, H&E (**b**). Note the rounded nuclei; oxyphilic, fluffy cytoplasm; and prominent nucleoli

membrane irregularities and intranuclear pseudoinclusions. The dominant oncocytic nodules show prominent oxyphilic changes as well, with rounded nuclear membranes, focally prominent nuclei, and infiltration of the nodule with lymphocytes (Fig. 1b; case 3, Table 2).

Molecular Findings

Genotyping for *BRAF*, *KRAS*, *NRAS*, and *HRAS* mutations

The dominant nodules and papillary carcinomas (from those with concomitant PTC) were screened for *BRAF* mutations using D-HPLC, a highly sensitive method for detecting a wide range of mutation types, including point mutations, insertions, and deletions [3, 4, 27].

The D-HPLC profiles for *BRAF* amplicons from papillary thyroid carcinomas arising in association with dominant nodules in lymphocytic thyroiditis are shown (Fig. 2). Of the 17 dominant nodules analyzed, none of them had a mutation in *BRAF*. However, in the nine cases of DN with associated papillary carcinomas, three of these cases showed *BRAF* aberrations (Fig. 2; Table 2). Patients 1 and 2 both had the common V600E mutation; in the case of patient 1, two separate foci of papillary carcinoma had this mutation (indicating two primary lesions or one intrathyroidal metastasis). Patient 7 had a small deletion/substitution in exon 15 (VKWRV600–604E). No mutations of *KRAS* (exons 1 and 2), *NRAS* (exons 1 and 2), or *HRAS* (exons 1 and 2) were found in dominant nodules or associated papillary carcinoma.

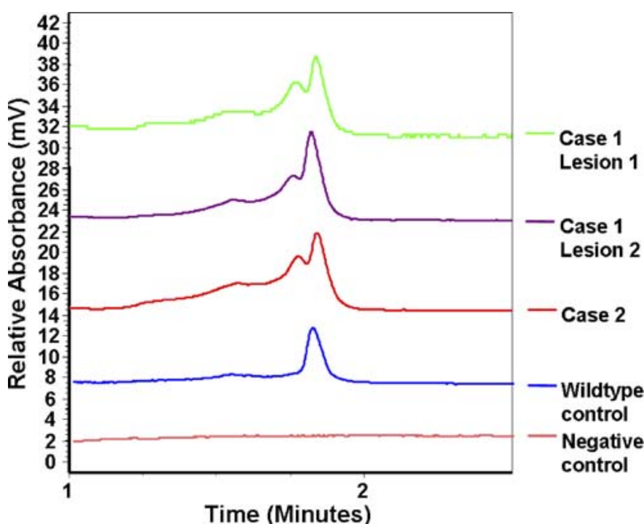

Fig. 2 D-HPLC profile for *BRAF* exon 15 (56.3°C). Analyses are shown for two foci of papillary thyroid carcinoma from one patient and another focus from a second patient. Note the *second peak*, indicating the presence of an aberrant *BRAF* allele

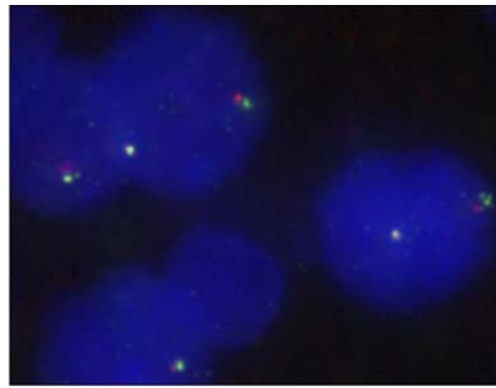

Fig. 3 Dual-color FISH break-apart probes for *RET* rearrangement. The colocalization of *red* and *green signals* indicates absence of *RET* rearrangement

RET-PTC1 and RET-PTC3

All 17 dominant nodules were negative for *RET-PTC1* and *RET-PTC3* translocation using break-apart probes (Fig. 3). In addition, a bring-together *RET-H4* FISH assay was negative in all dominant nodules studied, as well as associated papillary carcinomas. Cases were subsequently re-evaluated for *RET-PTC1* and *RET-PTC3* translocations using an reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay. Again, all dominant nodules and carcinomas were negative for *RET-PTC1* and *RET-PTC3* translocations.

Discussion

Hashimoto thyroiditis presents a number of challenges in diagnosis. The clinical presentation is variable, and the thyroid, on exam, may reveal multiple nodules of variable sizes. There has been a longstanding association of Hashimoto thyroiditis with carcinomas of the thyroid [15, 22, 23, 29], although definitive molecular links have remained elusive. Multinodularity in HT presents a clinical challenge: which nodule to biopsy and how many nodules to biopsy [16, 30]. In addition, in the setting of Hashimoto thyroiditis, fibrosis, lymphocytic infiltration, and oncocytic (Hürthle cell) changes can lead to a certain degree of atypia, for which neoplasia and metaplasia are in the differential. These changes can confound fine-needle aspirate interpretation [6, 34].

A Korean group showed activation of the RET/PTC–RAS–BRAF pathway in papillary carcinomas and oncocytic cells of Hashimoto thyroiditis, as compared with normal thyroid tissue, by immunohistochemical scoring of the expression of RET and nuclear-localized RAS [8]. However, the authors found no mutation in *BRAF* or *NRAS* in the oncocytic areas of HT [8]. More recently, another Korean group studied the association of *BRAF* mutations in

patients with papillary thyroid carcinoma with and without Hashimoto thyroiditis, and within their cohort of 101 PTC patients, 37 (36.6%) had concurrent HT. Of these HT-associated PTC patients, 27 (72.9%) had a *BRAF* mutation vs. 61 (95.3%) of non-HT PTCs [10].

In another recent study, Sargent and colleagues investigated the role of *BRAF* mutations in carcinomas associated with HT and in non-neoplastic areas showing nuclear atypia [25]. They noted that few of the HT-associated PTCs had *BRAF* mutations, and there were no *BRAF* mutations associated with uninvolved adjacent atypical HT epithelium. Within HT, there are often partially encapsulated nodules showing oncocytic metaplasia and varying degree of nuclear changes, including grooves and clearing [1, 9]. In the context of HT, there may also be a lymphocytic infiltrate within the nodule. These changes may be confused with carcinoma and are difficult to assess by hematoxylin and eosin stain alone.

As is often the case, thyroid lesions that are difficult to classify by fine-needle aspiration are challenging to classify even following excision [25]. Here, we have investigated molecular changes in 17 of 28 dominant nodules identified in 345 consecutive thyroidectomies for a clinical diagnosis of Hashimoto thyroiditis. Nodules with obvious features of carcinoma were excluded from the dominant nodule group but investigated alongside lesions that were not diagnostic of papillary carcinoma in the same patient.

No mutations in *BRAF* were identified in any of the dominant nodules investigated. Of the nine patients with concomitant papillary carcinomas (52.9% of investigated cases), four lesions in three patients harbored *BRAF* abnormalities. Thus, despite reports of the coincidence of carcinomas and Hashimoto thyroiditis, dominant nodules with atypical features do not appear to represent a precursor lesion.

RET translocations are common in papillary thyroid carcinomas, and levels of *RET* oncogene expression correlate with clinical behavior [2, 24]. Additionally, there have been conflicting reports as to whether RET-PTC fusion gene products are expressed in HT and whether these might serve as an initiating event for tumorigenesis in HT [5, 23, 28, 35]. It has been hypothesized that *RET-PTC* fusion genes facilitate immunoaberrant oncogenesis in HT [5]. Among 17 dominant nodules, we found no association of HT with *RET-PTC* fusions, although, in view of the evidence for such fusions in previously published HT studies, examination of a larger cohort may be of merit.

Mutations of RAS have been found in up to 25% of follicular-patterned thyroid cancers and even in benign follicular adenomas [11, 31, 33], with most aberrations occurring in *NRAS* [33]. In our study, we found one follicular carcinoma in the setting of HT with a dominant nodule (3.6%). No mutation in *NRAS*, *HRAS*, or *KRAS* was found in either the carcinoma or the dominant nodule in that case, nor were *RAS* mutations found in papillary carcinomas associated with dominant nodules.

In summary, we examined 17 dominant nodules in Hashimoto thyroiditis and found no mutations in *BRAF*, *NRAS*, *KRAS*, or *HRAS*. In addition, we did not find evidence for *RET* fusion oncogenes, which were interrogated using both FISH and RT-PCR methods. Our findings suggest that dominant nodules of Hashimoto thyroiditis are not premalignant lesions, even when there is cytologic atypia. A multimodality approach toward diagnosis in thyroid nodules of HT is the best determinant of malignant potential, with efficient and effective use of cytology, histology, immunohistochemistry, and molecular diagnostics.

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