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Frequency of Germline *PTEN* Mutations in Differentiated Thyroid Cancer

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Background: Differentiated thyroid cancer (DTC) is seen in 3%–10% of individuals carrying a germline *PTEN* mutation. Patients with *PTEN* mutations are at risk for additional neoplasms as are their affected offspring. However, the frequency of *PTEN* mutations among DTC cases has not been systematically analyzed. The objective of this study was to determine the frequency of *PTEN* mutations in an unselected group of patients with DTC and to identify whether additional clinical features might indicate the need for referral for genetic counseling and possible testing.
Methods: We collected personal medical and family history information, head circumference data, and blood from 259 consecutively identified clinic-based patients with DTC, unselected for personal or family history. Individuals were categorized for diagnostic criteria for Cowden syndrome (CS) using the 2009 National Comprehensive Cancer Network (NCCN) guidelines and underwent germline *PTEN* mutation analysis.
Results: Two of the 259 patients (0.8%), with both follicular thyroid carcinoma and macrocephaly, were found to carry a germline mutation in the *PTEN* gene. The *PTEN* mutation frequency in unselected cases of follicular thyroid carcinoma was 4.8%.
Conclusion: The frequency of germline pathogenic *PTEN* mutations in an unselected series of patients with DTC is relatively low, but it is enriched by considering follicular histology and macrocephaly. These results suggest that by adding head circumference to the clinical assessment, thyroid cancer specialists can more effectively identify patients needing referral for cancer genetic services.

Introduction

Differentiated thyroid cancer (DTC) including follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), and their subtypes account for over 80% of all thyroid cancers diagnosed annually. DTC can be seen as part of several hereditary cancer syndromes, including Cowden syndrome (CS, MIM 158350). CS is an autosomal dominant disorder caused by germline mutations in the *PTEN* gene and is part of the *PTEN* hamartoma tumor syndrome (1–7). Individuals with CS have an increased risk for nonmalignant tumors as well as specific malignancies, including breast cancer, DTC, and endometrial cancer (8–11). The lifetime risk of DTC in CS is between 3% and 10% (9,10,12). FTC is the most common histologic subtype in CS; however, PTC with classical or follicular variant histologies has also been described (10,13,14). Diagnostic criteria for CS have been developed to assist clinicians with the identification of patients who might benefit from cancer genetic risk assessment and genetic testing (11,15,16).

Although DTC is a common component of CS, the prevalence of germline *PTEN* mutations among patients with apparently sporadic DTC has not been systematically studied. In addition, it is not clear which features of CS in patients with thyroid cancer might be most predictive of a *PTEN* mutation. Here, we report our findings in a series of consecutively identified clinic-based patients with DTC, unselected for personal or family history tested for germline *PTEN* mutations.

Materials and Methods

Subject recruitment

The subjects were 259 consecutively enrolled patients with DTC visiting a multidisciplinary thyroid tumor clinic between August 2006 and September 2007. Individuals were eligible for an IRB-approved research study on individuals with thyroid cancer and completed detailed medical history and family history questionnaires. Participants were also asked to donate a blood sample for genetic research. Head circumference

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(HC) was collected on individuals using standard procedures (17) either at their initial enrollment visit or later. Histopathological classification was done according to the 2004 World Health Organization classification (18).

Individuals were categorized for diagnostic criteria for CS using the 2009 National Comprehensive Cancer Network (NCCN) guidelines (see Table 1). All information regarding the presence or absence of clinical features of CS was extracted from the self-reported medical history form, medical record, or both. Pathology reports were obtained for all 259 thyroid cancer diagnoses. In the 17 individuals meeting CS criteria, all major and minor criteria were confirmed with pathology reports when applicable and/or medical records, with the exception of 1 case of fibrocystic breast disease and 2 cases of uterine fibroids in three patients (please refer to Table 2).

PTEN *mutation analysis*

Germline genomic DNA was subjected to polymerase chain reaction (PCR)-based *PTEN* mutation scanning of all nine exons and flanking intronic regions using a combination of denaturing gradient gel electrophoresis (DGGE) as previously described (19) and high-resolution melt curve analysis using the LightScanner Technology (20). LightScanner primers defining the nine exons of *PTEN* were designed (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/thy) for high-resolution melt analysis using the LightScanner primer design software (version 1.0; Idaho Technology, Inc.).

All PCRs were performed in 96-well PCR plates (Bio-Rad Laboratories). PCR was performed in a 10 μL volume using 0.08 μL of Platinum Taq from Invitrogen (5 U/μL), 1.0 μL Invitrogen PCR buffer, 0.5 μL of 50 mM $MgCl_2$, 4.0 μL each of 1 mM each dNTP, 1.0 μL of 10$\times$LCGreenPlus solution, 0.2 μM final concentration each of primers, and 25 ng of template DNA. PCR conditions are 95°C for 2 minutes, followed by 37 cycles of 95°C for 30 seconds, Tm (see Supplementary Table S1) for 30 seconds, with a final heteroduplex formation step of 95°C for 30 seconds followed by 25°C for 30 seconds. The PCR products and their associated melting curves were then analyzed on the 96-well LightScanner (Idaho Technology, Inc.) using special 96-well plates for high-resolution melt analysis. The LightScanner Technology allows for analysis of high resolution melting from 70°C to 98°C. The resulting melting curves and their associated amplicons were analyzed using a second standard LightScanner software (version 2.0). DGGE or LightScanner melt curve variant-positive amplicons were independently amplified from a separate template and sub-

Table 1. 2009 NCCN Guidelines Cowden Syndrome Diagnostic Criteria

Criteria	
Pathognomonic criteria	LDD—adult Mucocutanous lesions: Trichilemmomas, facial Acral keratoses Papillomatous lesions
Major criteria	Breast Cancer Thyroid Cancer (papillary or follicular) Macrocephaly ($\geq$97th percentile) Endometrial cancer
Minor criteria	Other structural thyroid lesions (e.g., adenoma, multinodular goiter) Mental retardation (i.e., IQ $\leq$75) Gastrointestinal hamartomas Fibrocystic disease of the breast Lipomas Fibromas Genitourinary tumors (e.g., uterine fibroids, renal cell carcinoma) or Genitourinary structural malformations Uterine fibroids
Operational diagnosis in an individual:	Any of the following: 1. Mucocutanous lesions alone if (a) There are six or more facial papules, of which three or more must be trichilemmoma, or (b) Cutaneous facial papules and oral mucosal papillomatosis, or (c) Oral mucosal papillomatosis and acral keratoses, or (d) Palmoplantar keratoses, six or more 2. Two or more major criteria, but one must include macrocephaly or LDD; or 3. One major and three minor criteria; or 4. Four minor criteria.
Operational diagnosis in a family where one individual is diagnostic for CS:	1. One pathognomonic criterion; or 2. Any one major criterion with or without minor criteria; or 3. Two minor criteria; or 4. History of Bannayan–Riley–Ruvalcaba syndrome

Source: NCCN, 2009 (15).
CS, Cowden syndrome; LDD, Lhermitte-Duclos disease.

Table 2. Clinical Characteristics of 17 Patients Meeting 2009 NCCN Diagnostic Criteria for Cowden Syndrome

	Major CS criteria[a]			*Minor CS criteria*						
Gender/race	*PTEN result*	*Thyroid cancer histology*	*Macrocephaly (HC in cm)*	*Benign thyroid*	*GI hamartomas*	*FCBD*	*Lipomas*	*Fibromas*	*GU tumors/defects*	*Uterine fibroids*
F/African American	R15S[b]	FTC	Y (58.5)	N	Y	N	N	N	N	N
F/Caucasian	R130Q[b]	FTC	Y (62)	Y	U	N	N	N	N	N
M/Caucasian	Negative[b]	PTC	Y (60.25)	N	N	N	N	N	N	N
F/Caucasian	Negative[b]	PTC	Y (60.25)	Y	U	N	N	N	N	U
M/Caucasian	Negative[b]	PTC, FV	Y (60)	N	N	N	Y	N	N	NA
F/African America	Negative[b]	PTC, FV	N (56.5)	N	N	Y	N	N	Y	Y
F/Caucasian	Negative[b]	PTC, FV	N (55.5)	Y	N	Y	Y	N	N	Y (nc)
F/Caucasian	Negative[b]	PTC, FV	N (54.5)	N	N	Y	Y	N	N	Y
M/Caucasian	Negative[c]	microPTC	Y (63.5)	Y	N	N	N	N	N	NA
M/Caucasian	Negative[c]	FTC, OV	Y (61)	Y	N	N	N	N	N	NA
M/Caucasian	Negative[c]	PTC	Y (60)	N	N	N	Y	N	N	NA
M/Caucasian	Negative[c]	PTC, tall cell	Y (60)	N	N	N	N	N	N	N
F/Caucasian	Negative[c]	microPTC	Y (59)	N	N	N	N	N	N	N
F/Caucasian	Negative[c]	FTC, OV	N (56.5)	Y	N	Y	N	N	N	Y
F/Caucasian[d]	Negative[c]	PTC	N (56)	N	Y	Y	N	N	N	Y (nc)
F/Caucasian	Negative[c]	FTC	N (55.5)	N	Y	Y	N	Y	N	N
F/Caucasian	Negative[c]	PTC, FV	N (55.5)	Y	N	Y (nc)	N	N	N	Y

[a]No patient met pathognomonic CS criteria or had breast or endometrial cancer.
[b]Genetic testing by denaturing gradient gel electrophoresis.
[c]Genetic testing by LightScanner.
[d]Patient had single trichilemmoma confirmed by pathology as well as multiple keratoses.

HC, head circumference; GI, gastrointestinal; FCBD, fibrocystic breast disease; GU, genitourinary; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; OV, oncocytic variant; fv, follicular variant; Y, yes; N, no; U, unknown M, male; F, female; NA, not applicable; nc, per patient report only, not confirmed with medical records.

Table 3. Clinical and Demographic Features of 259 Differentiated Thyroid Cancer Cases

Gender	*n* (%)
Female	198 (76.4)
Male	61 (23.6)
Race	*n* (%)
Caucasian	244 (93.1)
Asian	7 (2.7)
African American	4 (1.9)
Other	4 (2.3)
Mean age at diagnosis	41.0 (range 12–88 years)
Histologic subtype (WHO, 2004)	*n* (%)
PTC	
Classic type	144 (55.6)
Follicular variant	34 (13.0)
Other variant[a]	38 (14.7)
FTC	
Minimally or widely invasive	25 (9.7)
Oncocytic type	17 (6.6)
Anaplastic	1 (0.4)
Total	259 (100)

[a]Includes micropapillary carcinoma.
WHO, World Health Organization.

jected to semi-automated Sanger sequencing using 3730xl DNA analyzer (Applied Biosystems). The promoter region was directly sequenced for all samples as previously described (21).

Results

Demographic and clinical information is shown in Table 3. Medical and family history questionnaires were completed by all but one subject. This individual's information was extracted from the medical record only. HC was available on 212 of the 259 subjects.

The mean age at first diagnosis of thyroid cancer was 41.0 years (range 12–88). Most patients had PTC, classic type (55.6%) or PTC, follicular variant (13.0%). Forty-two (16.2%) individuals had FTC. There were thrice as many women as men; consistent with the known increased female to male ratio seen in DTC (22).

Of the 212 individuals with complete clinical data, 17 (8.0%) individuals met the NCCN diagnostic criteria for CS (Table 2). Ten of the 212 had macrocephaly (occipitofrontal diameter $\geq$the 97th percentile), and this feature combined with a diagnosis of DTC meets CS clinical criteria. An additional seven individuals met CS criteria by having one major criterion (DTC) and three minor criteria.

Two individuals were found to carry disease-causing mutations in the *PTEN* gene. The first individual had FTC, macrocephaly, and multiple hamartomatous gastrointestinal polyps. The second individual had FTC, macrocephaly, and multinodular goiter. Thus, of 42 patients with FTC, 2 (4.8%; 95% CI = 0.6%–16.2%) were found to have pathogenic germline *PTEN* mutations as compared with none of the remaining 216 (0%; 95% CI = 0%–1.7%) with PTC ($p = 0.026$, two-tailed Fisher exact test). An additional four individuals had one of three variants of uncertain significance within the *PTEN* promoter (one subject each with −929G>A or −733G>A, two subjects with −1084C>T), and one individual had a synonymous substitution (c.234C>T, p.Thr78Thr) in exon 4. None of these five individuals met the clinical criteria for CS.

Conclusion

We tested 259 consecutive DTC cases unselected for clinical features or a family history consistent with CS for germline mutations in the *PTEN* gene and found two deleterious mutations, for an overall mutation frequency of 0.8% (95% CI = 0%–2.8%). When considering follicular histology only, which has been shown to be more closely associated with a diagnosis of CS (11) than papillary histology, the mutation frequency was 4.8% (95% CI = 0.6%–16.2%). None of the patients with PTC, even those with follicular variants, carried a *PTEN* mutation; and this difference was statistically significant ($p = 0.026$, two-tailed Fisher exact test).

Of the 212 individuals with HC data, 17 (8.0%) met diagnostic criteria for CS, including the two individuals who tested positive for a *PTEN* mutation, both of whom had FTC. Of those meeting CS criteria, the mutation frequency was 11.8% (2/17). This is lower than the 81% mutation frequency published by Marsh *et al.* (3) in a series of 37 individuals meeting the International Cowden Consortium criteria. This difference may suggest that some criteria, or their combination, are less predictive of germline mutations than others.

In the present series, 3 of the 17 individuals meeting diagnostic criteria, including the two mutation-positive patients, met them based on having FTC and macrocephaly, both of which are major clinical criteria for CS. This suggests that HC, at least in adults with FTC, may be one of the more easily obtained and predictive clinical features of a *PTEN* mutation. Many of the minor features of CS, such as fibrocystic breast disease and uterine fibroids, may not be routinely gathered in a typical medical history intake, may require a review of pathology records to confirm the diagnosis, are common in the general population, and are relatively nonspecific for CS. This can make the CS diagnostic criteria rather complicated and cumbersome. HC, in contrast, is a quick and easy measurement that can be performed on all patients.

This study has several weaknesses. First is the small number of mutation-positive cases. With only two *PTEN*-positive mutation results, it was not possible to explore multivariate predictors of the presence of a mutation or to make strong conclusions for individual predictors. Another potential weakness is the use of two different initial genetic scanning methods, LightScanner technology and DGGE. When mutation scanning technologies are used to prescreen samples, their accuracy should be considered. DGGE generally has a mutation detection rate of $\sim$80% (23,24). DGGE is highly sensitive and specific when the primers and GC clamps are carefully optimized. When this can be achieved, the accuracy is >99% (25). The LightScanner relies on high-resolution melt curve analysis technology by allowing for mutation scanning using the unique dsDNA binding dye "LCGreen," which is able to saturate DNA molecules. The binding of the dye is disrupted in the presence of a heteroduplex, and the resulting variation in fluorescence is detected by Lightscanner as the heteroduplex melts. Head-to-head comparison of 3 month's worth of samples by both scanning technologies was performed before this study with 100% concordance (C.E., unpublished data). Indeed, mutations, variations, and known

polymorphisms identified in this study were found using this method and all mutations were confirmed by DNA sequencing. In addition, the methods used here could not have detected large genomic deletions. However, only ~5% or less of CS cases are due to such deletions (21,26).

Third, individuals in this study did not have a detailed examination for features of CS. However, we feel that this represents the "real world" situation in a multidisciplinary thyroid cancer clinic, in which healthcare providers will not have the time and in most cases the expertise to evaluate each patient for CS. In addition to thyroid cancer, both patients who were mutation-positive had macrocephaly, which is a major feature of CS and is seen in up to 80% of patients with this condition (27). HC is an easily obtainable measurement and, when considered in the presence of DTC, increased the mutation detection rate significantly, from 0.8% to 20% (2 mutation positives out of 10 individuals with macrocephaly), in the present series.

Lastly, our HC data were incomplete. Consequently, there may be more individuals in the study who meet the CS diagnostic criteria. Estimates of the population frequency of macrocephaly are in the order of 2%–3% (28), and, therefore, it is unlikely that many of the 47 individuals without HC data were missed. It is unclear what normal HC range should be used for adult populations being evaluated for CS. The CS consortium and 2009 NCCN criteria define macrocephaly as an occipitofrontal diameter $\geq$the 97th percentile; however, no absolute cut-off values are provided within the criteria. Therefore, depending on which reference set is used, the proportion of individuals being classified as macrocephalic will vary. For the purposes of this study, we opted to use the HC data as published by Roche *et al.*, similar to the new 2010 NCCN criteria published after our study was completed (29,30). In this population, smoothed percentiles suggest a 97th percentile of ~60 cm for adult men and ~58 cm for adult women. Several factors that may influence HC are typically not taken into account when defining the normal HC distribution, such as ethnicity, height, and weight (29–31). Although the development of clinically accurate adult HC charts is beyond the scope of this investigation, it may have clinical relevance when adults are being assessed for macrocephaly and associated syndromes such as CS.

We demonstrated that the frequency of germline pathogenic *PTEN* mutations in an unselected series of patients with DTC is relatively low, but it is enriched by considering follicular histology and macrocephaly. By considering FTC histology alone or the combination of FTC and large HC, clinicians can identify a subset of patients with DTC most likely to benefit from referral for cancer genetic services, where a more thorough evaluation by a geneticist can be performed before consideration of *PTEN* testing. Since individuals with CS have a 35%–50% lifetime risk of breast cancer and an increased risk of other benign and cancerous lesions, early identification of at-risk individuals could lead to early screening and diagnosis for the patients and their at-risk family members.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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