Genetic Variants Implicate Dual Oxidase-2 in Familial and Sporadic Nonmedullary Thyroid Cancer

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Abstract

Highly penetrant hereditary thyroid cancer manifests as familial nonmedullary thyroid cancer (FNMT), whereas low-penetrance hereditary thyroid cancer manifests as sporadic disease and is associated with common polymorphisms, including rs965513[A]. Whole-exome sequencing of an FNMT kindred identified a novel Y1203H germline dual oxidase-2 (DUOX2) mutation. DUOX2Y1203H is enzymatically active, with increased production of reactive oxygen species. Furthermore, patients with sporadic thyroid cancer homozygous for rs965513[A] demonstrated higher DUOX2 expression than heterozygous rs965513[A/G] or homozygous rs965513[A]-negative patients. These data suggest that dysregulated hydrogen peroxide metabolism is a common mechanism by which high- and low-penetrance genetic factors increase thyroid cancer risk.

Significance: This study provides novel insights into the genetic and molecular mechanisms underlying familial and sporadic thyroid cancers.

Introduction

Thyroid cancer is the most common endocrine malignancy in the United States, resulting in 62,450 new diagnoses and 1,950 deaths in 2015 (1). Compared with other common cancers, thyroid cancer displays a high degree of heritability with first-degree relatives having a 5.5-fold increase in relative risk of disease (2). This increased thyroid cancer risk results from both high penetrance rare variants and low penetrance common variants (3–8). Hereditary cancer syndromes associated with medullary thyroid cancer have been well described; however, the genetic lesions associated with nonmedullary thyroid cancer heritability are less well defined. Familial nonmedullary thyroid cancer (FNMTC) is defined as thyroid cancer of follicular cell origin in two or more first-degree relatives in the absence of other cancer predisposition syndromes (9) and is thought to account for 3% to 10% of all thyroid cancers (10). FNMT families display a mix of benign and malignant thyroid tumors with an earlier age of onset and a higher incidence of multifocal disease compared with patients with sporadic thyroid cancer (10, 11). Germline mutations in HABP2, SRRM2, and FOXE1 have been reported in FNMT kindreds (3–5); however, whether all of these mutations truly cause FNMT remains controversial (12–14).

In contrast to FNMT, low-penetrance hereditary thyroid cancer has been associated with multiple common SNPs (6–8). The rs965513[A] allele, which confers the greatest relative risk for the development of thyroid cancer (6, 7), resides within an enhancer element controlling expression of FOXE1 (15, 16), a thyroid-specific transcription factor that regulates several genes including thyroid peroxidase (TPO), thyroglobulin (TG), the sodium-iodide symporter (SLC5A5), and dual oxidase-2 (DUOX2; ref. 17). The risk allele is associated with decreased expression of FOXE1 and siRNA knockdown of FOXE1 causes altered expression of DUOX2, TPO, TG, and SLC5A5 in vitro (17).

Using whole-exome sequencing, we identified a novel Y1203H germline DUOX2 mutation in an FNMT kindred. DUOX2 generates hydrogen peroxide (H2O2), which is used by TPO to iodinate tyrosyl residues on TG for thyroxine (T4) and 3,3’,5-triiodothyronine (T3) synthesis, and loss-of-function mutations in DUOX2 cause congenital hypothyroidism (18). We report that DUOX2Y1203H is functionally active and is highly expressed in normal thyroid tissue, potentially contributing to increased tumorigenesis. Strikingly, DUOX2 expression is increased in patients with rs965513[A] homozygosity, consistent with a model in which DUOX2-mediated dysregulation of H2O2 metabolism underlies both high- and low-penetrance hereditary thyroid cancers.
Materials and Methods

Subjects were recruited at the Penn State College of Medicine. The study was approved by the Penn State College of Medicine Institutional Review Board. Written informed consent was obtained from all participants whose DNA and tumor tissues were analyzed.

Nucleic acid extraction, whole-exome capture, and next-generation sequencing

Germline DNA was extracted from whole blood or saliva using a Qiagen QIAsymphony nucleic acid isolation robot. Qiagen AllPrep DNA/RNA Mini Kits were used to extract nucleic acids from tumor and normal tissue stored in RNA later at –80°C (Thermo Fisher Scientific) or liquid nitrogen. Additional details are provided in Supplementary Methods.

Genomic DNA was sheared into approximately 200-bp fragments and processed into Illumina-compatible sequencing libraries with a PrepX ILM DNA Library Kit (WaferGen). Sheared DNA (500–1,000 ng) was processed into Illumina-compatible sequencing libraries with a PrepX ILM DNA Library Kit (WaferGen) on an Apollo 324 instrument (WaferGen). Libraries were uniquely bar coded, amplified, and subjected to multiplex SeqCap EZ Exome V3.0 (Roche) hybridization and capture. Libraries were sequenced on an Illumina 2500 (Illumina Inc.) using 2 × 100 bp reads in high-output mode. Sequence data have been deposited at the European Genome–phenome Archive (EGA), which is hosted by the European Bioinformatics Institute (EBI) and the Centre for Genomic Regulation, under accession number EGAS00001003782. Further information about EGA can be found on https://ega-archive.org. “The European Genome–phenome Archive of human data consented for biomedical research” (http://www.nature.com/ng/journal/v47/n7/full/ng.3312.html). Additional details about library preparation and sequencing are provided in Supplementary Methods.

Sequencing data pipeline and variant filtering

Next-generation sequencing data were processed according to Genome Analysis Toolkit (GATK) best practices recommendations using GATK v2.7.4 (19–21). Variants within the exome capture region ± 100 bp were identified using the GATK Haplotype Caller module v3.3.0 (20, 21). Additional details are provided in Supplementary Methods. Germline variants were filtered using Golden Helix SNP and Variation Suite v8.1.5 (Golden Helix, Inc.) according to the following criteria: (i) segregation with an autosomal dominant mechanism of inheritance; (ii) SNPs produced a nonsynonymous variation in a defined exon; (iii) SNPs were present in <0.5% of the population encompassed by the 1,000 Genomes Project Phase 1, the dbSNP build 137 common variants database (http://www.ncbi.nlm.nih.gov/SNP; ref. 22), the NHLBI GO Exome Sequencing Project build 138 database (23), and the Exome Aggregation Consortium database version 0.3.1 (24); and (iv) SNPs had a variant quality score >20 and read depth >10.

We next filtered for variants in genes that had a previously known function reported in PubMed and for those highly expressed in thyroid tissue. Tissue-specific gene expression data were derived from Illumina Body Map 2.0, GTEx, and Uhlén’s lab experiments in the EMBL-EBI Expression Atlas Database (25–27). Varnants were retained if they occurred within a gene differentially expressed in thyroid tissue across all three data sets. Differential expression was defined by $E_i > 1.5Q_3 - Q_1 + Q_3$ where $E_i$ represents expression of gene $i$ in thyroid tissue, $Q_3$ represents the third quartile expression value for gene $i$ across all tissues, and $Q_1$ represents the first quartile gene expression value for gene $i$ across all tissues. Variants occurring in genes where the raw expression value was less than the mean $Q_2$ value for all genes in thyroid tissue across all three databases were also excluded. PolyPhen-2 (28) scores were used to identify variants with potential functional impact, and variants with a score <0.3 were excluded. For the remaining variants, literature searches were conducted to identify any potential function for impacted genes in the thyroid gland; only genes directly implicated in thyroid gland physiology were included. Further testing required that candidate SNPs be absent in an unaffected and unrelated spouse and present in an additional affected family member.

Somatic mutation detection

Somatic mutations in tumor exomes were detected using MuTect v1.1.7 (29). To reduce the incidence of false-positive mutation calls due to sequencing artifacts, alignment errors, and rare germline mutations, the “panel of normals” flag was implemented as described (30) using white blood cell whole-exome sequence data from 50 patients with no known hematologic malignancy. Details regarding the “panel of normals” can be found in Supplementary Methods. Filtered Variant Call Format files were annotated using TabAnno v2.13 (31) and somatic mutations were confirmed by visual inspection in Integrative Genomics Viewer (32).

Protein modeling and alignment

Modeling of the DUOX2 peroxidase domain (NCBI accession NP_054799.4) was performed using SWISS-MODEL (33). Amino acid sequences of DUOX2 or the orthologous protein were obtained for the following species: Homo sapiens (NCBI accession NP_054799.4), Pan troglodytes (NCBI accession XP_009427327.1), Macaca mulatta (NCBI accession XP_014997623.1), Canis lupus familiaris (NCBI accession XP_013964824.1), Bos taurus (NCBI accession XP_018087708.1), Mus musculus (NCBI accession NP_808278.2), Rattus norvegicus (NCBI accession NP_077055.1), Drosophila melanogaster (NCBI accession NP_001259968.1), and Caenorhabditis elegans (NCBI accession NP_490684.1). Protein alignment was performed using Clustal Omega (34).

PCR, Sanger sequencing, and targeted deep sequencing

Sanger sequencing was used to verify variants identified by next-generation exome sequencing. PCR amplification of regions spanning DUOX2Y1203-rrs965513, BRAFV600, KRASQ61, HRASQ61, and NRASQ61 was conducted using primers shown in Supplementary Table S1. Details regarding PCR conditions are described in Supplemental Methods. Sanger sequencing was conducted by GENEWIZ LLC. Mutation status was determined by visual inspection of fluorescence trace files of capillary electrophoresis. Targeted deep sequencing libraries of gel-purified BRAFV600 and KRASQ61 PCR products were constructed using the KAPA hyper prep kit (KAPA Biosystems). Libraries were sequenced on an Illumina MiSeq instrument generating 2 × 250 bp reads. Adapter sequences were trimmed and reads were aligned to the hg19 reference genome using the FASTQ Toolkit and BWA Aligner apps on the Illumina BaseSpace website (http://basespace.illumina.com).
Plasmids and mutagenesis

The vectors EX-NEG-M02, human DUOX2 (EX-E1601-M02), and human DUOX2 (EX-H0633-M02) were purchased from Genecopoeia. The DUOX2Y1203H expression vector was prepared using Applied Biosystems (Thermo Fisher Scientific) and human DUOXA2 (EX-E1601-M02) were purchased from Genecopoeia. The DUOX2Y1203H expression vector was prepared using Applied Biosystems (Thermo Fisher Scientific) TaqMan Gene Expression Master Mix and TaqMan Probes targeting FOXE1 (catalog no. Hs00916085-S1), DUOX2 (catalog no. Hs00204187-m1), TPO (catalog no. Hs0892518-m1), TG (catalog no. Hs00794359-m1), and GAPDH (catalog no. Hs02758991-G1). Reactions were analyzed on an Applied Biosystems QuantStudio 12K Flex Instrument (Thermo Fisher Scientific). Expression of each target was determined as fold expression relative to GAPDH averaged over three independent replicates. Differences in gene expression relative to rs965513 genotype were computed using the Kruskal–Wallis rank sum test as implemented by the "stats" package in R v3.2.2 (35).

Cell culture and stable transfections

Cos7 (CRL1651, ATCC) cells were cultured in DMEM (ATCC) with 10% fetal bovine serum (ATCC) under 5% CO2/95% air at 37°C. To produce stable cell lines, Cos7 cells were transfected with wild-type human DUOX2A in a cytomegalovirus-driven expression vector (pReceiver-M02) or empty vector using Lipofectamine 3000 (Thermo Fisher Scientific) transfection reagent according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were replated in medium with Geneticin (G418; Thermo Fisher Scientific; 500 μg/mL) for selection. After 2 weeks of selection, cells were pooled, expanded, and maintained with Geneticin (G418; 250 μg/mL).

Reactive oxygen species measurements

The multiplex ROS-Glo H2O2 Assay (Promega) and the Amplex Red Hydrogen Peroxide/Peroxidase Assay (Thermo Fisher Scientific) were used to measure extracellular H2O2, whereas intracellular reactive oxygen species (ROS) were measured using a DCFDA ROS Detection assay kit (AbCam). For all assays, Cos7 cells stably expressing DuoxA2 were plated in 96-well plates and grown to 70% to 80% confluence according to the manufacturer’s protocol. Cells were then transiently transfected with the EX-NEG-M02 empty vector, DUOX2 vector, or the DUOX2Y1203H vector using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacture’s protocol. Twenty-four hours after transfection, the ROS and cell viability assays were performed according to the manufacturer’s instructions. Relative luminescence was determined using a SpectraMax 13 Multiplate reader (Molecular Devices). All experiments were performed in triplicate. ROS production for each transfection condition was standardized to the empty vector transfection negative control. Statistical differences in ROS production were calculated using Student’s t-test as implemented in Microsoft Excel (Microsoft Corporation).

Similar protein expression across experiments was confirmed using semiquantitative Western blotting of cells transfected in parallel. Briefly, cell extracts were prepared in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma), and protein concentrations were determined by the BCA method. Equal quantities of protein input (30 μg) were separated by 4% to 12% Nupage Tris-Bis gel electrophoresis with SeeBlue Plus 2 Protein Standard (Invitrogen) under reducing conditions and transferred to an Immobilon-P transfer membrane (EMD Millipore). Immunoblots were incubated with DUOX2 S-12 (MABN787; EMD Millipore) diluted 1:1,000, GAPDH diluted 1:1,000 (sc-98501; Santa Cruz Biotechnology), and DUOX2A diluted 1:100 (sc-98501; Santa Cruz Biotechnology). Blots were processed using a ScanLater kit with appropriate secondary antibodies according to the manufacturer’s instructions (Molecular Devices, LLC) and quantified using ImageJ (36).

HIC

Slides were prepared by 5-μm sections of formalin-fixed paraffin-embedded tissue. Representative sections were stained with hematoxylin and eosin and fitted with glass coverslips for imaging. Remaining samples were deparaffinized using standard procedures and antigen retrieval was performed by incubation in 10 mmol/L sodium citrate, pH 6.0 with 1 mmol/L EDTA at 95°C for 20 minutes. Samples were incubated with mouse anti-DUOX2 S-12 monoclonal antibody (0.5 mg/mL, diluted 1:1,000; MABN787; EMD Millipore) at 4°C overnight. Slides were then incubated with unlabeled ImmPRESS goat antimouse-HRP secondary antibody (Vector Laboratories) for 30 minutes at room temperature, washed, and developed with diaminobenzidine (DAB; Vector Laboratories). Slides were counterstained with hematoxylin prior to being fitted with glass coverslips for imaging. Slides were reviewed by a board-certified head and neck pathologist (Joshua I. Warrick).

Results

Identification of a novel missense mutation associated with FMTC

The proband (Fig. 1A, patient III.2) presented with multifocal micropapillary thyroid carcinoma at 46 years of age. She had an extensive family history of thyroid cancer. The proband’s maternal grandmother (Fig. 1A, patient I.2), who ultimately succumbed to the disease, had three children (II.2, II.3, and II.4) all of whom were diagnosed with thyroid cancer. Individual II.2 has been treated for highly aggressive, recurrent papillary thyroid cancer (PTC) at our institution (Fig. 1A). Individuals II.1 and II.2 had four children, all of whom were affected: two with papillary thyroid cancer (proband and III.4), and two with nodular thyroid disease (III.3 and III.5; Fig. 1A). The disease status of individuals III.6, III.7, and III.8 is not known. The proband (III.2) has two children (IV.1 and IV.2), one of whom (IV.1) was diagnosed with a benign thyroid nodule by ultrasound examination at age 19 years (Fig. 1A). Importantly, no patient in this pedigree exhibited clinical symptoms of hypothyroidism. The prethyroidectomy thyroid-stimulating hormone (TSH) level for the
proband patient was 4.4 μIU/mL (reference range: 0.35–5.5 μIU/mL), although preoperative TSH levels were not available for the other individuals in the study. This pedigree is consistent with autosomal dominant hereditary papillary thyroid cancer (Fig. 1A).

Germline DNA from five affected individuals (Fig. 1A, dashed lines) was subjected to whole-exome sequencing. After filtering, missense variants were identified in three genes: DUOX2, IFT140, and WDR72 (Table 1). Because of its central role in thyroid physiology, we focused our analysis on DUOX2. A single, heterozygous A to G variant at chr15:45,389,898, predicted to cause a Y1203H substitution in DUOX2 (Table 1), was identified in all affected individuals (Fig. 1A). Sanger sequencing confirmed the presence of the variant in all affected individuals but not in an unaffected family member (Fig. 1A, patient III.1).

The probability of this mutation occurring in all affected family members is approximately 1.6%. Analysis of the dbSNP database (22) revealed that the prevalence of this mutation in the general population is approximately 1/138,000 individuals, indicating that DUOX2_Y1203H is an extremely rare mutation. No hereditary cancer predisposition syndromes known to cause thyroid cancers were detected in affected individuals (Supplementary Table S2). Furthermore, all sequenced

Figure 1.
Familial nonmedullary thyroid cancer associated with a novel mutation in DUOX2. A, Pedigree of a kindred with highly penetrant autosomal-dominant FNMT across four generations. The key on the lower right indicates disease status for each patient. The proband patient is indicated by the arrow and the age of each patient at diagnosis is shown to the right. The allele status of Chr15:15:45,389,898 is shown below selected patients and was confirmed by examining electropherograms from Sanger sequencing. Patients subjected to whole-exome sequencing are indicated by the dashed lines. B, A cartoon depiction of DUOX2 and its cognate maturation factor DUOXA2. The star indicates the approximate location of the Y1203H mutation. C, Amino acid sequence alignments from the DUOX2 protein of nine vertebrate and invertebrate species demonstrate that Y1203 is highly conserved among mammals. Amino acid positions from human DUOX2 are shown below the alignment and the red box indicates Y1203. Transmembrane domains are shown as blue shading. D, Cos7 cells were stably transfected with DUOXA2 (Cos7-DUOXA2) or empty vector (Cos7-EV) and transiently transfected with empty vector (EV), wild-type (WT) DUOX2, or DUOX2_Y1203H. ROS production was measured as a function of cell viability using three assays: RosGlo (extracellular ROS), Amplex Red (extracellular ROS), and DCFDA (intracellular ROS). The graphs show the mean relative fluorescence value of three independent experiments, with error bars indicating SEM. Data were normalized to the baseline established by EV transfection, which was set to a value of 1. *P < 0.05. E, A representative Western blot from the experiment outlined in D showing similar expression of wild-type DUOX2 and DUOX2_Y1203H in Cos7-DUOX2 cells. Transient transfection of wild-type DUOX2 or DUOX2_Y1203H into Cos7-EV cells resulted in minimal expression of DUOX2 or DUOX2_Y1203H. Expression of wild-type or mutant DUOX2 relative to GAPDH is shown below each lane.
patients were negative for the HABP2_G346D, FOXE1_A246G, and SRRM2_A346E polymorphisms previously implicated in FNMT (Supplementary Table S2; refs. 3, 4).

The DUOX2 protein consists of an extracellular N-terminal peroxidase domain; seven transmembrane domains containing four invariant histidines, which may be coordination sites for two nonidentical prosthetic heme moieties (37); two EF-Hand calcium-binding domains; and intracellular C-terminal FAD- and NADPH-binding domains (Fig. 1B). Expression, proper localization, and function of DUOX2 are dependent on its cognate maturation factor, DUOXA2 (Fig. 1B), which interacts with the extracellular “A” loop of DUOX2 (37). The Y1203H mutation is located within the fifth transmembrane domain, which is distant from the enzymatic active site and the DUOX2A interaction domain (Fig. 1B), but is highly conserved across mammalian species (Fig. 1C).

Functional analysis of DUOX2_Y1203H

Previous reports have described DUOX2 mutations, predominantly in the extracellular peroxidase domain or the intracellular calcium-binding domain, resulting in loss of function and congenital hypothyroidism (37). We therefore tested whether the DUOX2_Y1203H variant disrupts the H2O2 and ROS-generating capacity of the enzyme. Because DUOX2 expression and H2O2 synthesis requires coexpression of DUOX2A (37), we generated a Cos7 cell line that stably transfected with empty vector (Fig. 1E), confirming that DUOX2_Y1203H expression is dependent upon DUOXA2 as expected (37). These data demonstrate that DUOX2_Y1203H contains a functional peroxidase domain capable of producing H2O2 and suggest that DUOX2_Y1203H increases extracellular ROS compared with wild-type DUOX2.

DUOX2 expression in normal thyroid and thyroid tumor tissue

We next sought to determine the effect of DUOX2_Y1203H on DUOX2 expression in normal thyroid and thyroid tumor histology by comparing tissue samples from the proband patient (Fig. 1A, patient III.2) and a patient with sporadic thyroid cancer. Upon hematoxylin and eosin staining, nontumor tissue from the proband patient was normal in appearance (Fig. 2A) and was similar to non-tumor thyroid tissue from the patient with sporadic thyroid cancer (Fig. 2B). Histologic examination of the proband’s thyroid gland revealed two distinct tumors (Fig. 2A): a 7-mm tumor with follicular variant papillary thyroid cancer architecture (Fig. 2A, Micro-PTC 1), and a 4-mm tumor with classical papillary thyroid cancer architecture (Fig. 2A, Micro-PTC 1), and a 4-mm tumor with classical papillary architecture (PTC; Fig. 2A, Micro-PTC 2). A single tumor with classical papillary architecture was identified in the thyroid gland excised from the patient with sporadic thyroid cancer (Fig. 2B).

DUOX2 is highly expressed in thyroid follicular cells (25, 38). To determine the effect of the Y1203H substitution on DUOX2 expression, we performed IHC to compare DUOX2 expression between the proband and the patient with sporadic thyroid cancer. Antibody specificity was confirmed by Western blotting (Fig. 1E). Thyroid follicular cells stained strongly for DUOX2 in normal tissue from the proband (Fig. 2A, α-DUOX2) and a patient with unrelated sporadic thyroid cancer with wild-type DUOX2 (Fig. 2B, α-DUOX2). The proband’s Micro-PTC-1 lesion exhibited strong DUOX2 staining, although expression was grossly reduced in Micro-PTC-2 (Fig. 2A), suggestive of clonally distinct tumors arising within a single individual. The tumor isolated from the patient with sporadic PTC exhibited DUOX2 expression

### Table 1. Whole-exome sequencing variant filtering protocol

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<th>Filtering step</th>
<th>Mean coverage depth</th>
<th>Number of variants</th>
</tr>
</thead>
<tbody>
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<td>1. Variants identified</td>
<td></td>
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</tr>
<tr>
<td>• Patient II.2</td>
<td>107.3x</td>
<td>644,267</td>
</tr>
<tr>
<td>• Patient III.2</td>
<td>68.1x</td>
<td>541,180</td>
</tr>
<tr>
<td>• Patient III.3</td>
<td>90.4x</td>
<td>608,064</td>
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<td>• Patient III.4</td>
<td>112.7x</td>
<td>1,728,519</td>
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<tr>
<td>• Patient III.5</td>
<td>94.4x</td>
<td>1,826,547</td>
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<tr>
<td>2. Heterozygous variants present in all affected family members</td>
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<td>3. Nonsynonymous variants in protein coding exons</td>
<td></td>
<td>1,560</td>
</tr>
<tr>
<td>4. Variants in &lt;0.5% of population represented by Exome Variant Server, dbSNP, and 1000 genomes databases</td>
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<td>5. Genotype quality &gt;20 and read depth &gt;20</td>
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<tr>
<td>6. Variant in a highly expressed gene that is differentially expressed in thyroid tissue</td>
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<tr>
<td>7. PolyPhen-2 score &gt; 0.300</td>
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Nucleotide</th>
<th>Amino acid</th>
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<td>DUOX2</td>
<td>Chr15:45,389,898</td>
<td>c.3607T&gt;C</td>
<td>p.Y1203H</td>
<td>H2O2-generating NADPH oxidase, highly expressed in thyrocytes</td>
</tr>
<tr>
<td>IFT140</td>
<td>Chr16:1,574,650</td>
<td>c.3044A&gt;C</td>
<td>p.H1015P</td>
<td>Flagellar transport gene. Mutation results in retinal dystrophy or cystic kidney disease.</td>
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<tr>
<td>WDR72</td>
<td>Chr15:53,994,442</td>
<td>c.1458C&gt;A</td>
<td>p.D486E</td>
<td>Mutations associated with amelogenesis imperfecta include Val491Ile, S783Ter, W978Ter, and S953Vfs. No evidence for specific function in thyroid, may be involved in parathyroid function.</td>
</tr>
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</table>
similar to adjacent normal thyroid tissue (Fig. 2B). Together, these data indicate that DUOX2Y1203H heterozygosity does not dramatically reduce DUOX2 expression, although expression of DUOX2 may vary depending upon the differentiation pattern of thyroid tumors.

To determine whether DUOX2Y1203H was associated with DNA mutagenesis in the thyroid gland, we profiled the mutational spectrum of tumor tissue and adjacent normal tissue from the proband and the patient with sporadic thyroid cancer using whole-exome sequencing. The MicroPTC-1 tumor from the proband was positive for the KRASQ61R oncogenic driver mutation whereas the Micro-PTC2 tumor was positive for the BRAFV600E driver mutation (Fig. 2A). The mutual exclusivity of oncogenic driver mutations in the two tumors was confirmed by Sanger sequencing and targeted next-generation sequencing to a depth of >15,000× (Fig. 2A). Sequence analysis of the sporadic tumor revealed a single BRAFV600E mutation (Fig. 2B). The exclusivity BRAFV600E oncogenic driver mutation was confirmed by targeted next-generation sequencing (Fig. 2B).

The rs965513[A] thyroid cancer risk polymorphism is associated with increased DUOX2 expression

The mechanism through which the rs965513[A] polymorphism influences risk of nonmedullary thyroid cancer is not known. We hypothesized that altered expression of FOXE1, and consequently DUOX2, in patients with the rs965513[A] polymorphism may underlie sporadic thyroid cancer susceptibility. To test this hypothesis, we genotyped 64 patients with thyroid cancer for the rs965513 allele using Sanger sequencing and quantified expression of FOXE1, DUOX2, and other thyroid-specific genes in normal thyroid tissue by quantitative PCR. The A/G genotype was the most common (n = 32, 50%), followed by the G/G genotype (n = 17, 27%) and the A/A genotype (n = 15, 23%). Expression of FOXE1 was lower in the high-risk A/A group than in the G/G and A/G genotypes, although this difference did not reach statistical significance (Fig. 3A). Higher expression of DUOX2 (P = 0.033), TPO (P = 0.031), TG (P = 0.029), and SLC5A5 (P = 0.014) was found in the A/A group (Fig. 3B–E), consistent with decreased FOXE1 promoter activity (8).

Discussion

Few genes have been identified as bona fide causes of FNMT, partially due to the low prevalence of familial disease. The estimated prevalence of thyroid cancer in the United States is 0.14% (1), with FNMT accounting for 3% to 10% of cases (10). Thus, only 0.0042% to 0.014% of individuals in the general population are likely to be affected by FNMT in their lifetime. Our analysis of a single highly penetrant pedigree using exome sequencing identified a novel germline Y1203H missense variant in DUOX2, which may underlie sporadic thyroid cancer susceptibility.
in DUOX2 that segregated as an autosomal dominant thyroid cancer phenotype. The DUOX2_Y1203H variant seems to be a very rare mutation that has been identified in only 1 of 138,000 individuals in the dbSNP database (22), consistent with its role as a causative mutation in a rare condition such as FNMTC.

Other germline variants, including HABP2_G534E, SRRM2_A346F, and FOXE1_A248G (3–5), have been reported to cause FNMTC. The prevalence of HABP2_G534E SNP at 1% to 5% (13, 14, 22, 39) and FOXE1_A248G at 5.3 of 1000 individuals (22) is not consistent with the predicted prevalence of FNMTC at 1 of approximately 10,000 individuals in the general population (10). The SRRM2_A346F polymorphism is more rare with a predicted prevalence of 6 of 10,000 individuals (22), however, the mechanism by which this mutation induces thyroid cancer remains unknown (5). Given that FNMTC is an uncommon disease, true causative mutations may be poorly represented, even in large datasets, consistent with the apparent rarity of DUOX2_Y1203H (22). Furthermore, the observation that all cases of FNMTC reported thus far are associated with distinct germline mutations suggests that the FNMTC phenotype may be the common endpoint resulting from a variety of genetic lesions.

A potential role for DUOX2 in thyroid cancer has recently been suggested (40). The DUOX2_Y1203H variant is a compelling potential cause of FNMTC because DUOX2 is the major enzyme that generates H2O2 required for thyroid hormone synthesis in the thyroid gland. Hydrogen peroxide may be involved in carcinogenesis via multiple mechanisms including oxidative DNA damage, cell signaling, cell proliferation, regulation of gene expression, and cell senescence and apoptosis (40). Interestingly, H2O2 directly damages both DNA and RNA, leading to DNA and RNA mutagenesis and increased turnover (41).

Increased DNA mutagenesis in association with DUOX2_Y1203H is supported by the finding that the two thyroid tumors in the proband are genetically independent and have distinct oncogenic driver mutations (Fig. 2A). Multifocality is thought to be more common among patients with FNMTC than among patients with sporadic thyroid cancer (10, 11). Tumorigenesis is a complex process and should therefore be a rare event (42, 43). The presence of multiple genetically independent tumors within a single thyroid gland raises the intriguing possibility that patients with multifocal thyroid cancer, particularly those with a family history of thyroid cancer, may have a genetic predisposition toward thyroid tumorigenesis. The precise mechanism underlying DUOX2_Y1203H-mediated tumorigenesis remains to be determined; however, in vitro experiments suggest that Y1203H increases H2O2 production (Fig. 1D). Interestingly, two assays that measure extracellular ROS (ROS-Glo and Amplex Red) indicate that DUOX2_Y1203H increases extracellular ROS levels compared with wild-type DUOX2, whereas measurement of

Figure 3.
Effect of rs965513 genotype on thyroid-specific gene expression. Sanger sequencing was used to determine the rs965513 genotype for 64 patients with papillary thyroid cancer. Messenger RNA expression levels were determined for FOXE1 (A), DUOX2 (B), TPO (C), TG (D), and SLC5A5 (E) and are expressed as log-fold expression relative to GAPDH controls. Each point indicates gene expression from an individual patient, determined as the mean of three independent experiments. Patients were divided by rs965513 genotype as shown below each graph. Box plots indicate median expression values, with hinges at the upper and lower quartile and whiskers extending to 1.5× the interquartile range. *, P<0.05.
intracellular ROS using DCFDA demonstrated similar ROS production between wild-type and mutant DUOX2. These data suggest that the DUOX2 Y1203H mutation may result in dysregulated ROS production at the plasma membrane; however, further studies are required to evaluate this possibility. Although the differences in ROS production that we observed were small and measured over a short time period (24 hours), changes in ROS metabolism that persist over many years may have cumulative biological effects that contribute to thyroid tumorigenesis, which occurs over the course of decades.

Homozygous or compound heterozygous mutations that compromise DUOX2 enzymatic activity and result in congenital hypothyroidism are found primarily in the N-terminal extracellular peroxidase-like domain and in the EF hand domains (18, 38, 44). By contrast, the tyrosine at position 1203 resides in the extracellular peroxidase-like domain and in the EF hand domains (18, 38, 44). The Y1203 residue is near two transmembrane heme moieties that coordinate heme binding, although the effect of altered heme binding on protein function is not known. Future studies will be required to determine the precise mechanism underlying the contribution of the DUOX2 Y1203H missense variant to increased susceptibility to nonmedullary thyroid cancer.

Although it is possible that the Y1203H mutation alters the interaction between DUOX2 and DUOXA2, several observations argue against this hypothesis. First, the interaction between DUOX2 and DUOXA2 is mediated by the N-terminal extracellular A-loop of DUOX2, which is remote from Y1203 (37). In addition, the location of Y1203 within a transmembrane domain (37) suggests that Y1203H is not likely to participate in protein–protein interactions. Finally, protein–protein interactions between DUOX2 and DUOXA2 are required for the maturation and function of DUOX2 (37). Our data indicate that DUOX2 Y1203H is enzymatically active, thereby providing indirect evidence for an intact interaction between DUOX2 Y1203H and DUOXA2.

The identification of DUOX2 as a potential cause of FNMTIC led us to investigate whether DUOX2 dysregulation may also play a role in sporadic PTC. The rs965513 variant is a common polymorphism associated with 3.1-fold increased risk of developing thyroid cancer in homozygous individuals (6). We found that 23% of the patients with sporadic PTC analyzed in this study were homozygous for rs965513[A], more than double the expected 11% to 12% prevalence of rs965513[A] homozygosity in the general population (6, 22). We found that individuals homozygous for rs965513[A] expressed higher levels of DUOX2, TPO, TC, and SLC5A5 (Fig. 3) than heterozygous rs965513[A/G] or homozygous-reference rs965513[G] individuals. These data are consistent with a potential mechanism for the rs965513[A] allele to increase thyroid cancer susceptibility via enhanced DUOX2 expression and H2O2 production. Similar mechanisms may therefore underlie highly penetrant FNMTIC and some cases of PTC caused by low-penetrance risk factors including rs965513[A].

We note the significant intragroup variability in gene expression. Given that each data point represents a mean of three independent experiments, it is likely that there is true biological variability in gene expression among the population, although the underlying reasons are not known. Possibilities for variability in thyroid-specific gene expression may include undiagnosed thyroid pathology, such as hypothyroidism or autoimmune thyroid disease, and environmental effects.

In summary, we used an unbiased approach to identify a novel germline mutation in DUOX2 associated with highly penetrant FNMTIC. Combined with evidence that the FOXE1 rs965513[A] polymorphism also increases DUOX2 expression, our data suggest that dysregulation of proteins involved in H2O2 metabolism may be a common mechanism underlying common high- and rare low-penetrance genetic factors that increase thyroid cancer susceptibility. This observation has translational implications for screening, chemoprevention, and development of therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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