THYROID
Volume 30, Number 3, 2020

DOI: 10.1089/thy.2019.0561

Thyroid Carcinomas That Occur in Familial Adenomatous Polyposis Patients Recurrently Harbor Somatic Variants in *APC*, *BRAF*, and *KTM2D*

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Background: Familial adenomatous polyposis (FAP) is a condition typically caused by pathogenic germline mutations in the *APC* gene. In addition to colon polyps, individuals with FAP have a substantially increased risk of developing papillary thyroid cancer (PTC). Little is known about the events underlying this association, and the prevalence of somatic ''second-hit'' mutations in *APC* is controversial.
Methods: Whole-genome sequencing was performed on paired thyroid tumor and normal DNA from 12 FAP patients who developed PTC. Somatic mutation profiles were compared with clinical characteristics and previously sequenced sporadic PTC cases. Germline variant profiling was performed to assess the prevalence of variants in genes previously shown to have a role in PTC predisposition.
Results: All 12 patients harbored germline mutations in *APC*, consistent with FAP. Seven patients also had somatic mutations in *APC*, and seven patients harbored somatic mutations in *KMT2D*, which encodes a lysine methyl transferase. Mutation of these genes is extremely rare in sporadic PTCs. Notably, only two of the tumors harbored the somatic *BRAF* p.V600E mutation, which is the most common driver mutation found in sporadic PTCs. Six tumors displayed a cribriform–morular variant of PTC (PTC-CMV) histology, and all six had somatic mutations in *APC*. Additionally, nine FAP-PTC patients had rare germline variants in genes that were previously associated with thyroid carcinoma.
Conclusions: Our data indicate that FAP-associated PTCs typically have distinct mutations compared with sporadic PTCs. Roughly half of the thyroid cancers that arise in FAP patients have somatic ''second-hits'' in *APC*, which is associated with PTC-CMV histology. Somatic *BRAF* p.V600E variants also occur in some FAP patients, a novel finding. We speculate that in carriers of heterozygous pathogenic mutations of tumor suppressor genes such as *APC*, a cooperating second-hit somatic variant may occur in a different gene such as *KTM2D* or *BRAF*, leading to differences in phenotypes. The role of germline variance in genes other than APC (9 of the 12 patients in this series) needs further research.

Keywords: familial adenomatous polyposis, *APC*, papillary thyroid cancer, cribriform–morular variant, whole-genome sequencing

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Introduction

In addition to colon polyps and colorectal cancer (CRC), patients with familial adenomatous polyposis (FAP) have an increased risk to develop extracolonic malignancies and benign conditions/tumors. Of particular interest is the frequent occurrence of papillary thyroid cancer (PTC), which is some 100 times more prevalent in FAP patients than in the general population (1). FAP is typically caused by germline mutations in the *APC* gene that result in a truncated APC protein and inevitably develops into CRC when somatic ''second-hit'' mutations in *APC* occur in colon cells (2). Previous studies have confirmed the existence of such ''second hits'' in the *APC* gene in a subset of thyroid cancers that occur in FAP patients, (3) but this remains controversial, as other reports have concluded that the *APC* gene is rarely somatically mutated in FAP-associated PTC (4–6).

This contradiction has not been resolved. FAP-associated PTCs often display a cribriform–morular variant (CMV) histology, which is otherwise extremely rare ($\sim$0.2% of all thyroid cancers) (7). Altogether, about half of all CMV-PTCs occur in FAP patients (7,8). In general, PTC is about three times more common in females than in males, and this ratio is even higher in PTC-CMV patients and FAP-associated PTCs (7). Molecular characteristics of PTC-CMV include mutations in the *CTNNB1* and/or *PIK3CA* genes and *RET*/PTC rearrangements. No oncogenic *BRAF* mutations have so far been reported in CMV-PTCs or FAP-associated PTCs; however, a comprehensive assessment of the somatic alterations that occur in FAP-associated PTCs has not been conducted.

To better understand the germline and somatic variants found in this unique tumor type and to try to learn more about the genetic mechanisms of thyroid cancer development in individuals with FAP-associated germline mutations in *APC*, we performed whole-genome sequencing of paired tumor and normal DNA from 12 FAP-associated PTC patients.

Materials and Methods

Patients

All patients with both FAP and PTC were selected from the PTC patient repositories at the Cleveland Clinic and the Helsinki University Hospital. Histological review of tumor sections was performed to confirm the presence of cancer in the resected thyroid. This study was limited to patients with germline *APC* mutations detected by clinically approved testing methods. All patients provided written informed consent, and studies were performed in accordance with the Declaration of Helsinki and approved by institutional review boards at both institutions.

DNA extraction and sequencing

DNA was extracted from paraffin-embedded thyroid tumors and adjacent normal tissue (patients 2, 4, 5, 7, 8, 9, 10, and 12) using QIAamp DNA FFPE Tissue Kits (Qiagen, Hilden, Germany) or from blood (patients 1, 3, 6, and 11) using a previously described nonenzymatic DNA extraction method (9). DNA samples were quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA), and fragment size was assessed using a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). All samples had average fragment sizes >1500 bp. Library preparation and paired-end genome sequencing were performed by Novogene (Beijing, China), using TruSeq Nano DNA HT Sample Preparation Kits (Illumina, San Diego, CA) and HiSeq 4000 instruments (Illumina, San Diego, CA). All samples were sequenced to >80 gigabytes of data.

Informatics

The quality of sequences was confirmed using FASTQC software. All samples had >99% of reads mapped, and all samples had >90% of bases with phred-scaled quality scores >30. Mapping was performed with Burrows–Wheeler Aligner to the Genome Reference Consortium Human Build 37. Samples were sorted with SamTools, duplicates were marked with Picard, and variants were called with GATK and then annotated with ANNOVAR. For somatic and germline variant analysis, paired .vcf files from each tumor and matching adjacent normal thyroid or blood sample were loaded into BasePlayer software (10). Variants present in the tumor sample and absent but covered in the paired germline DNA were considered somatic. Publicly available data from the American Association for Cancer Research Genomics Evidence Neoplasia Information Exchange (AACR-GENIE) and The Cancer Genome Research Atlas (TCGA) were accessed using cbioportal.org on August 19, 2019. Somatic variant signature analysis was performed using the R package DeconstructSigs (11), and the 30 signatures were described in the catalog of somatic mutations in cancer (COSMIC) mutational signatures (v2-March 2015). Somatic loss of heterozygosity (LOH) was determined using BasePlayer software (10) by comparing the allelic ratios of sequenced germline variants with their corresponding ratios in the matched tumor sample as described (12,13). A tumor to normal ratio of $\leq$0.6 or $\geq$1.67 was considered LOH, and a tumor to normal ratio of 0.6–0.8 or 1.25–1.67 was considered putative LOH.

Results

The 12 FAP-PTC patients in our cohort consisted of 4 males and 8 females (Table 1). Five patients displayed classic PTC histology (three males and two females), six patients had PTC-CMV (one male and five females), and one female had follicular variant of PTC. The number of tumors varied from one to two per individual and tumor sizes varied between 0.1 and 9 cm. All patients in our sample set were diagnosed with FAP before thyroid cancer, with an average age at FAP diagnosis of 25 years (range 12–44 years), and an average age at PTC diagnosis of 38 years (range 20–62 years). Patients were diagnosed with thyroid carcinoma on average 15 years after being diagnosed with FAP (range 1–46 years after FAP diagnosis). This cohort displayed characteristics typically associated with FAP, including CRC, ampullary cancer, and desmoid tumors.

Whole-genome sequencing was performed on paired tumor and normal DNA from all 12 cases. An oncoprint of the most commonly mutated genes is presented in Figure 1a. Seven patients had somatic ''second-hit'' mutations in *APC*, including one patient with clear somatic LOH of *APC* and two patients with putative LOH. The lysine methyl transferase gene *KMT2D* was also mutated in 7 of the 12 PTC

Table 1. Clinical Characteristics of the 12 Papillary Thyroid Cancer Patients with Familial Adenomatous Polyposis

Patient no.	*Sex*	*Histology*	*Age (FAP)*	*Age (thyroid cancer)*	*No. of thyroid tumors*	*Tumor size(s), cm*	*Ascertainment*	*Other FAP-related disease presentation*
1	Male	PTC-CMV	12	30	1	9	***	Desmoid tumors
2	Female	PTC-CMV	21	35	2	0.2 0.1	*	Desmoid tumors
3	Female	PTC-CMV	43	45	1	2.4	**	—
4	Female	PTC-CMV	33	35	2	0.9 0.3	*	Colon cancer
5	Female	PTC-CMV	18	43	2	0.9 0.2	* and **	—
6	Female	PTC-CMV	21	21	2	2 3	**	Desmoid tumors
7	Female	PTC	18	42	1	0.9	*	Unspecified extrathyroid cancer
8	Male	PTC	20	44	2	0.8 1	*	Desmoid tumors, adrenal adenoma
9	Female	PTC	16	62	1	0.4	* and **	Ampullary cancer
10	Male	PTC	23	28	1	1.6	*	Desmoid tumors
11	Female	PTC-FV	24	24	1	0.9	***	Desmoid tumors
12	Male	PTC	44	45	2	0.6 0.4	*	Desmoid tumors

*Annual ultrasound screening of FAP patients.
**Subjective symptoms.
***Computed tomography scan for unrelated reasons.
CMV, cribriform–morular variant; FAP, familial adenomatous polyposis; PTC, papillary thyroid cancer.

tumors, and another KMT2 family member, *KMT2C*, was mutated in three patients (four mutations).

The recurrently mutated genes in these samples were strikingly different from genes that are typically mutated in sporadic PTC, as evidenced by comparison with the AACR-GENIE and TCGA of PTC data sets (Supplementary Table S1). Specifically, mutations in *KMT2C*, *KMT2D*, and *APC* were only detected in <3% of sporadic cases. Conversely, *BRAF* is mutated in about 60% of all PTCs, and the vast majority of these mutations consist of the known cancer driver mutation *BRAF* p.V600E. However, in our sample set, only two cases harbored *BRAF* mutations (both p.V600E). Notably, neither of these samples had somatic *APC* mutations. *RET*/PTC translocations are also common in typical PTCs (14); however, in our cohort, there was no evidence of breakpoints between exons 11 and 12 in *RET*. Therefore, it is likely that none of the APC-associated PTCs we sequenced harbored *RET*/PTC or other *RET* translocations/inversions. We did not detect any somatic mutations in the *RAS* genes (*NRAS*, *HRAS*, and *KRAS*), which are recurrently mutated in sporadic PTC.

All clinically detected germline nonsense, frameshift, and insertion/deletion variants in the *APC* gene were validated in our genome sequencing. Three samples had complex *APC* germline mutations: patient 11 had a heterozygous ~23 Mb deletion of chromosome 5q that contained *APC*, patient 4 had a deletion of exons 15–16 (NM_000038, coding exons 14–15), and patient 1 had a C to T transition within intron 11 that results in incorporation of an out-of-frame pseudoexon and underlies FAP, as we have previously described (Table 2) (15). The remaining nine patients all had frameshift and nonsense germline mutations in the *APC* coding sequence, located between codons 471 and 1465 (Fig. 1b and Table 2). The ''second-hit'' somatic variants in *APC* were more spread out, and there was not an obvious correlation between the location of the germline mutation in *APC* and the somatic second hit, in contrast to reports that the location of the germline FAP-associated *APC* mutation can influence the position of the somatic *APC* variant in CRC secondary to FAP (Fig. 1b and Table 2) (16).

We examined the specific base pair substitutions in the mutations in these samples and compared them with described mutational signatures associated with different cancers and cancer subtypes (17). Most samples showed strong correlations with expression signatures 1 (pan-cancer deamination of 5-methylcytosine), 3 (failure of DNA double-strand break-repair by homologous recombination), 5 (pan-cancer with unknown etiology), 12 (unknown etiology signature found in liver cancer), and 20 (defective DNA mismatch repair) (Fig. 2a, b). The tumor of patient 6 exhibited a strong signal for mutation signature 6, which is seen in microsatellite unstable tumors that have DNA mismatch repair defects, and mutation signature 19, which is an unknown etiology signature found in pilocytic astrocytomas (Fig. 2c) (17–20). Notably, patient 6 did not have somatic mutations in *MLH1*, *PMS2*, *MSH2*, or *MSH6* and also did not have likely pathogenic germline variants in these genes.

Finally, we examined the germline variants in these samples for alleles that might play a role in thyroid cancer, specifically focusing on nonsynonymous variants in genes previously implicated in familial thyroid cancer (21). Because rare variants are more likely to be high-risk alleles for diseases than common variants, based on family studies (22), we only examined nonsynonymous variants with a population minor allele frequency less than 0.01 in the gnomAD database. We found that nine of the FAP-PTC patients had germline variants in 17 different genes, which were previously found to harbor variants associated with familial thyroid carcinoma (21) (Supplementary Table S2). Interestingly,

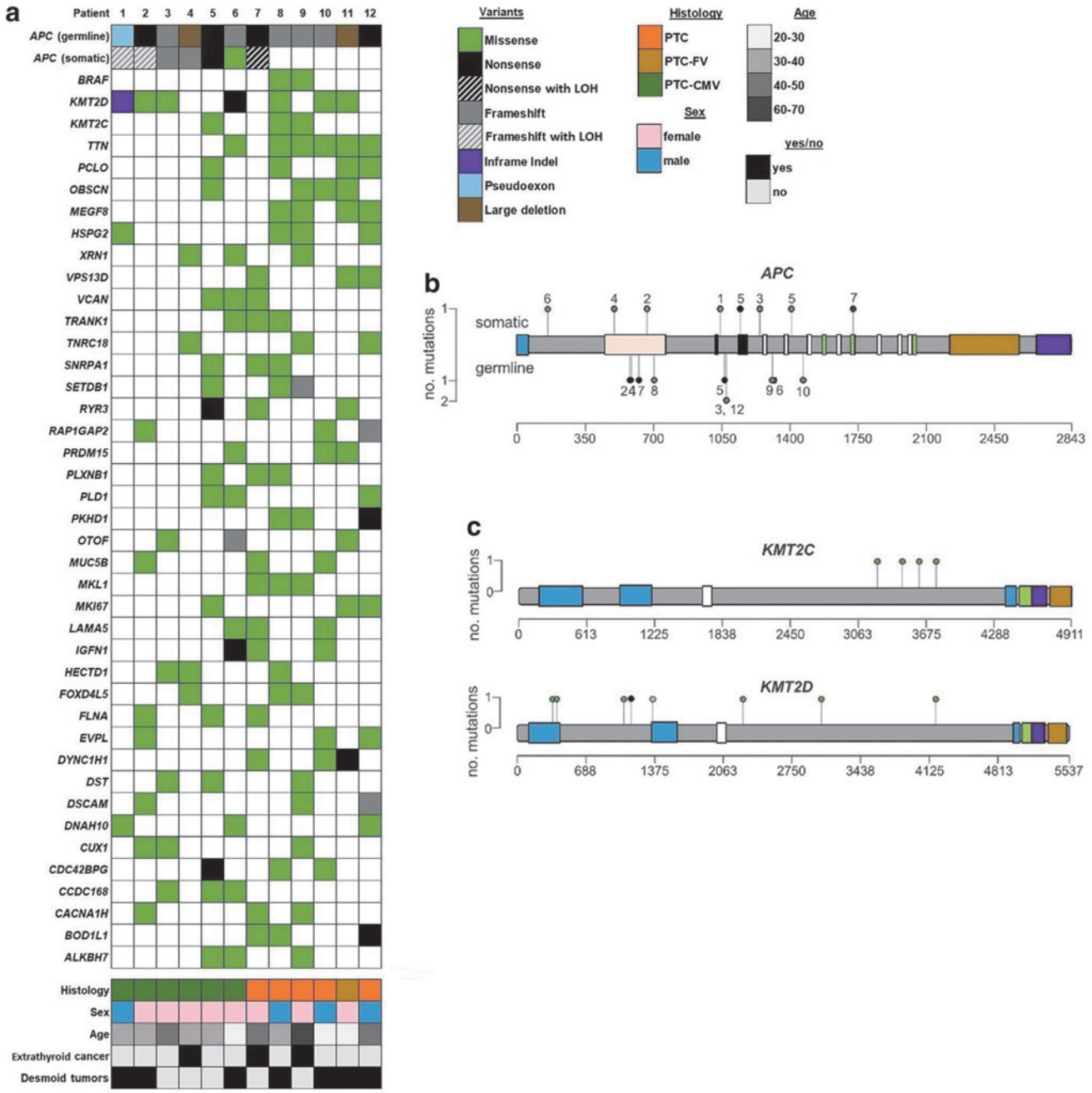

FIG. 1. Mutations and demographics of 12 FAP patients with PTC. (**a**) Oncoprint shows pathogenic germline *APC* variants and somatic nonsilent mutations in the indicated genes. Demographic and other clinical information is presented below the variants. (**b**) Lollipop plot shows all nonsilent somatic (top side) and germline (bottom side) variants in the *APC* coding sequence. Numbers adjacent to the mutation circles correspond to patient numbers. *APC* coding sequence is presented for NM_000038.6, and domains are colored as follows: blue, dimerization domain; tan, armadillo repeats; black, 15 amino acid repeats; white, 20 amino acid repeats; green, SAMP repeats; gold, basic domain; purple, EB1 binding. (**c**) Lollipop plot shows somatic mutations in *KMT2C* (NM_170606.3) and *KMT2D* (NM_003482.3). Domains are colored as follows: blue, plant homeodomain fingers; white, high mobility group box; green, FY-rich N-terminal domain; purple, FY-rich C-terminal domain; gold, SET methyl transferase domain. All variants are colored according to the legend. CMV, cribriform–morular variant; FAP, familial adenomatous polyposis; LOH, loss of heterozygosity; PTC, papillary thyroid cancer. Color images are available online.

there were several variants in the *RNF213* gene in three different FAP-PTC patients. *RNF213* has been found to be mutated in liver cancer (23), and all three of the patients (4,5,8) with *RNF213* variants had a strong signal for mutational signature 12 (Fig. 2a), which is linked to liver cancer. The p.R752L *FGD6* variant was found in two patients, one from the United States (patient 9) and one from Finland (patient 1). *FGD6* is located on chromosome 12q22, and this band has been observed to be amplified in thyroid adenomas and might also play a role in thyroid carcinomas as well (24).

Table 2. Notable Germline and Somatic Variants Identified in 12 Familial Adenomatous Polyposis–Papillary Thyroid Cancers

Patient no.	APC *(germline)*	APC *(somatic)*	BRAF *(somatic)*	KMT2C *(somatic)*	KMT2D *(somatic)*
1	c.1408+731C>T (p.Gly471Serfs*55)	c.3119_3149del (p.Arg1040Lysfs*6) and putative LOH	—	—	c.4059_4061del (p.Glu1354del)
2	c.1732G>T (p.Glu578*)	c.1995delA (p.Leu665Phefs*5) and LOH	—	—	c.1039C>T (p.His347Tyr)
3	c.3202_3205delTCAA (p.Ser1068Glyfs*57)	c.3729_3730delTC (p.Gln1244Lysfs*11)	—	—	c.12593G>A (p.Arg4198Gln)
4	c.1744_8532del (p.Glu582fs*14)	c.1490delT (p.Leu497Glnfs*9)	—	—	—
5	c.3183_3187delACAAA (p.Gln1062fs*)	c.3429T>A (p.Tyr1143*) and c.4195C>T (p.Arg1399Cys)	—	c.10681C>T (p.Pro3561Ser) and c.10235G>A (p.Arg3412Gln)	—
6	c.3927_3931delAAAGA (p.Glu1309Aspfs*4)	c.436G>A (p.Ala146Thr)	—	—	c.3415C>T (p.Gln1139*)
7	c.1873C>T (p.Gln625*)	c.5176G>T (p.Glu1726*) and putative LOH	—	—	—
8	c.2108_2109insG (p.Val704Serfs*2)	—	c.1799T>A (p.Val600Glu)	c.11137G>T (p.Ala3713Ser)	c.6797G>A (p.Gly2266Asp)
9	c.3920_3924delTAAAA (p.Ile1307Argfs*6)	—	c.1799T>A (p.Val600Glu)	c.9571C>T (p.Leu3191Phe)	—
10	c.4393_4394delAG (p.Ser1465Trpfs*3)	—	—	—	c.9136G>A (p.Glu3046Lys)
11	c.1-?_8532+?del (full gene deletion)	—	—	—	c.1168G>A (p.Val390Ile) and c.3190G>A (p.Val1064Ile)
12	c.3202_3205delTCAA (p.Ser1068Glyfs*57)	—	—	—	—

Mutations are reported for the following transcripts: *APC*, NM_000038.6; *BRAF*, NM_004333.5; *KMT2C*, NM_170606.3; *KMT2D*, NM_003482.3.
LOH, loss of heterozygosity; no., number.

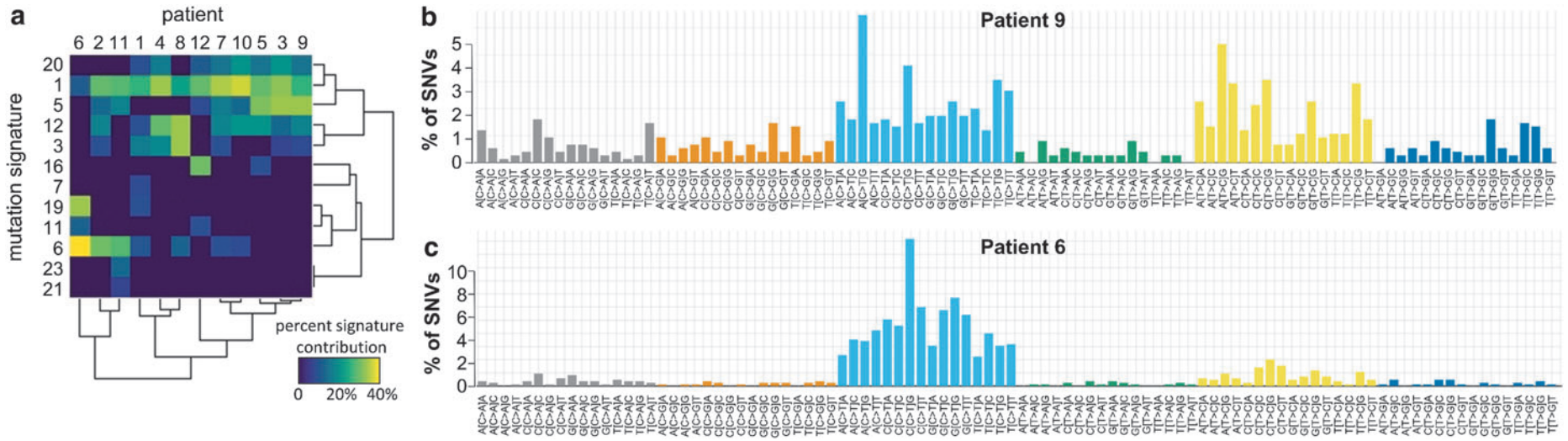

FIG. 2. Signatures of somatic mutations in thyroid cancer patients with FAP. (**a**) Heat map shows the relative contributions of each of the 30 COSMIC v2 mutational signatures for the 12 thyroid cancers in patients with FAP. Signatures that did not contribute to any patients are not shown. (**b**) The profile displaying the fraction of somatic mutations found in each of the 96 possible trinucleotide combinations of single nucleotide variants for a typical sample in this cohort (patient 9), whose tumor showed mutation consistent with signatures 1, 5, 12, and 20. (**c**) The mutation profile for patient 7, whose tumor was mostly composed of signatures 6 and 19, is consistent with signatures 1, 5, 12, and 20. COSMIC, catalog of somatic mutations in cancer; SNVs, single nucleotide variant. Color images are available online.

Discussion

It is surprising, although not entirely unexpected, that we detected somatic second hits in *APC* in over half of the FAP-PTC tumors we analyzed. Cetta *et al.* reported that somatic mutations in *APC* do not occur in FAP-associated PTC (4,25), but in contrast, somatic second-hit mutations in *APC* were previously reported by other groups (3,5,6). One explanation of this apparent contradiction is the recently improved sequencing methodology, and the failure of some researchers to examine the entire *APC* coding sequence.

Mechanistically, pathogenic nonsense and frameshift *APC* mutations lead to truncated APC protein products that are unable to interact with the cytoplasmic complex that mediates β-catenin degradation. Thus, the β-catenin/Lef/Tcf complex remains unchecked in the nucleus where it activates WNT signaling pathways responsible for enhanced cellular migration, proliferation, and loss of differentiation (26). Our finding is consistent with the idea that somatic second hits and/or LOH in *APC* further add cancerous properties to the cell and likely contribute to malignant transformation. Our identification of the somatic *BRAF* p.V600E mutation in two patients is a novel finding, as to our knowledge, no single case has been described in the literature where oncogenic *BRAF* mutations occur in either FAP-PTC or PTC-CMV (7,27–32). This implies that some PTCs arising in the context of germline pathogenic *APC* variants can share the same driver mutations as sporadic PTCs.

The mutual exclusivity of the somatic *APC* and *BRAF* mutations is consistent with different molecular subtypes of PTC occurring in different FAP patients. The tumors with *BRAF* p.V600E mutations displayed typical PTC histology and occurred in one male and one female. However, the patients with nonsilent somatic *APC* variants were almost entirely female (6 to 1, female to male), and in six of seven cases showed a PTC-CMV histology. Interestingly, the patient with a somatic *APC* mutation who did not have a PTC-CMV histology (patient 7) harbored the most 3′ *APC* mutation we detected. The mutated APC protein in patient 7 likely retains some beta-catenin binding ability, and we speculate this could explain that patient 7 did not have a CMV histology.

What does it mean that 58% and 33% of the PTC tumors in these FAP patients have somatic variants in the *KMT2D* and *KMT2C* genes, respectively? *KMT2D* and *KMT2C* are methyl transferase genes that encode important pieces of the COMPASS complex (33). Pathogenic somatic mutations in both of these genes have been detected in many different cancers, such as oropharyngeal squamous cell carcinoma, T cell lymphoma, bladder cancer, head and neck cancer, and breast and endometrial cancers but are extremely rare in sporadic PTC (34–37). Notably, *KMT2D* somatic variants have been shown to contribute to increased mutational burden and genome instability (38).

Prompted by the occurrence of *KMT2D* somatic variants, we looked for a biological link between *APC* and *KMT2D*, and to our surprise, it seems that *KMT2D* might also be involved in WNT signaling. *KMT2D* together with the *ALK* gene are connected with *CTNNB1* (β-catenin) (39), thus, it is no surprise that in some tumors somatic mutations occur in *KMT2D* instead of *APC*.

We speculate that the detected somatic variants in these genes (particularly *KMT2D*) might be important in the context of activated WNT signaling caused by FAP-associated germline *APC* mutations, and evidence of epigenetic dysregulation in these cases warrants further investigation. Our data implicate that the mutations in *KMT2D* may be cancer drivers in the patients in which they were observed.

The overall female to male ratio in our cohort is less skewed toward females than other reports of FAP-PTC patient demographics (7,40). For example, Lam *et al*. (7) reported that the female to male ratio in PTC-CMV is 31 to 1, whereas in our cohort there was only a 5 to 1 ratio of females to males among patients with PTC-CMV. However, we do acknowledge that the modest size of our cohort does not lend itself to definitive conclusions regarding sex ratios. Seven of the 12 patients had desmoid tumors, which is not surprising given that this a common feature of FAP patients (29,41–45).

Our study is the first that suggests mutations in genes other than *APC* can cooperate with the germline APC variant in FAP patients to drive thyroid cancer. Also, our findings provide new information on the genetic steps that participate in the carcinogenesis process. Our data are consistent with a model where the pathogenic germline *APC* variants act as ''gatekeepers'' in the thyroid. Some patients, almost always females, acquire somatic second hits in *APC* that drive a thyroid cancer with CMV histology. In other cases, oncogenic activating mutations somatically occur in *BRAF*, similar to sporadic thyroid cancers. In patients who lack clear driver mutations in *APC* or *BRAF*, an intriguing possibility is that somatic variants in other genes (e.g., *KTM2D*, *KMT2C*, and others) may act as cancer drivers in the thyroid. This concept postulates that a somatic heterozygous variant in a gene such as *KTM2D* can act as a trigger of the malignant transformation of a cell heterozygous for a pathogenic variant in another gene (i.e., *APC*) and is in line with the concept that multiple events contributing different cancerous properties to a cell need to occur in order for a malignancy to develop and proliferate (46).

It is striking that 9 of the 12 patients we sequenced harbored rare nonsynonymous mutations in 17 selected genes known to be associated with familial thyroid cancer. This is consistent with the concept that additional germline variants other than of *APC* can contribute to PTC formation in FAP patients. Further studies are necessary to unequivocally prove a causative role for the implicated germline variants in FAP-associated PTC and explore their interactions with the altered WNT signaling caused by pathogenic *APC* variants.

Acknowledgments

The authors would like to acknowledge: the patients who consented to provide material for this study; Jan Lockman and Barbara Fersch for administrative help; the OSUCCC Genomics Shared Resource and the Plant Microbe Genomics Facility for sequencing assistance; the OSUCCC Human Cancer Genetics Sample Bank for sample processing and storage; and the Ohio Supercomputer Center for computational resources.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This work was supported by the Leukemia Clinical Research Foundation, the Pelotonia fellowship program, the Jane and Aatos Erkko Foundation, the Finnish Cancer Organizations, the Sigrid Juselius Foundation, the Inkeri and Mauri Vänskä Foundation, the HiLIFE Fellows 2017–2020, the National Cancer Institute Grants P01CA124570 and P50CA168505, and The Academy of Finland Grant 294643.

Supplementary Material

Supplementary Table S1
Supplementary Table S2

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