Original Article

Thyroid carcinomas that occur in familial adenomatous polyposis patients recurrently harbor somatic variants in *APC*, *BRAF*, and *KTM2D*

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Abstract

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 carcinoma (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.Val600Glu mutation, which is the most common driver mutation found in sporadic PTCs.

Six tumors displayed cribriform-morular variant of PTC (PTC-CMV) histology, and all six of these had somatic mutations in *APC*. Additionally, 9 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 to 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

119 histology. Somatic *BRAF* p.Val600Glu variants also occur in some FAP patients, a
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121 novel finding. We speculate that in carriers of heterozygous pathogenic mutations of
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123 tumor suppressor genes such as *APC,* a cooperating second-hit somatic variant may
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125 occur in a different gene such as *KTM2D* or *BRAF*, leading to differences in
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127 phenotypes. The role of germline variance in genes other than APC (9 of 12 patients in this
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129 series) needs further research.
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Introduction

In addition to colon polyps and colorectal cancer, 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 colorectal cancer (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 (4,5,6). This contradiction has not been resolved.

FAP-associated PTCs often display cribiform-morular variant (CMV) histology, which is otherwise extremely rare (~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.

Material 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 non-enzymatic 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 > 1500bp. Library preparation and paired-end genome sequencing was performed by Novogene (Beijing, China), using Truseq Nano DNA HT Sample Preparation Kits (Illumina, San Diego, CA) and HiSeq 4000 instruments (Illumina, San Deigo, 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 done 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 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 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 to their corresponding ratios in the matched tumor sample as described (12,13). A tumor to normal ratio of ≤0.6 or ≥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 papillary thyroid carcinoma. Number of the tumors varies from 1 to 2 per individual

and tumor sizes varies between 0.1 cm and ? 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 colorectal cancer,

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 seven of the 12 PTC 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 1). 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.Val600Glu. However, in our sample set only two

cases harbored *BRAF* mutations (both p.Val600Glu). 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 ~23Mb 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, 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 colorectal cancer secondary to FAP (Fig. 1b, Table 2) (16).

We examined the specific base pair substitutions in the mutations in these samples and compared them to 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). Patient 6's tumor 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) (17,18,19,20). 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

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400 on family studies (22), we only examined nonsynonymous variants

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402 with a population minor allele frequency less than 0.01 in the gnomAD database. We

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404 found 9 of the FAP-PTC patients had germline variants in 17 different genes, which were

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406 previously found to harbor variants associated with familial thyroid carcinoma (21)

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408 (Supplementary Table 2). Interestingly, there were several variants in the *RNF213*

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410 gene in three different FAP-PTC patients. *RNF213* has been found to be mutated in liver

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412 cancer (23) and all three of the patients (4, 5, 8) with *RNF213* variants had a strong signal for

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414 mutational signature 12 (Fig. 2a), which is linked to liver cancer. The p.Arg752Leu *FGD6* variant

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416 was found in two patients, one from the USA (patient 9) and one from Finland (patient 1).

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418 *FGD6* is located on chromosome 12q22, and this band has been observed to be amplified

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420 in thyroid adenomas, and might also play a role in thyroid carcinomas as well (24).

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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.Val600Glu 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 (7,27,28,29,30,31,32). 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.Val600Glu mutations displayed typical PTC histology, and occurred in one male and one female. However, the patients with non-silent somatic *APC* variants were almost

entirely female (6 to 1, female to male), and in six of seven cases showed PTC-CMV histology. Interestingly, the patient with a somatic *APC* mutation who did not have 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 contribute to why patient 7 did not have 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 (34,35,36,37). Notably, KMT2D somatic variants have

been shown to contribute to increased mutational burden and genome instability (38).

Prompted by *KMT2D* somatic variants, we looked for biological link between *APC* and *KMT2D* genes, and to our surprise, it seems that also *KMT2D* might be involved in WNT signaling. *KMT2D* together with *ALK* gene are connected with *CTNNB1* (β-catenin) (Pinckney et al. 2018, Applied Cancer Research, 38:13), so it is not surprise that in some tumors instead of *APC,* somatic mutations occurs in *KMT2D.*

We speculate that the detected somatic variants in these genes (particularly KMT2D) might be important in the context of deactivated 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 towards females than other reports of FAP-PTC patient demographics (7,39). 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 twelve patients had desmoid tumors, which is not surprising given that this a common feature of FAP patients (29,40,41,42,43,44).

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 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 (45). To prove or disprove this scenario in FAP-associated thyroid cancer, a larger series of

cases will need to be studied, and gene functions and interactions carefully documented.

It is striking that 9 out 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 idea 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.

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The authors declare no conflict of interest.

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Table 1. Clinical characteristics of the 12 PTC patients with FAP

Patient no.	Sex	Histology	Age (FAP)	Age (thyroid cancer)	Number thyroid tumors	Tumor size(s)	Ascertainment	Other FAP-related disease presentation
1	Male	PTC-CMV	12	30	1	9 cm	Incidental	desmoid tumors
2	Female	PTC-CMV	21	35	2	0.2 cm 0.1 cm	Screening ultrasound	desmoid tumors
3	Female	PTC-CMV	43	45	1	2.4 cm	Mass in the neck, “self finding”	.
4	Female	PTC-CMV	33	35	2	0.9 cm 0.3 cm	Screening ultrasound	colon cancer
5	Female	PTC-CMV	18	43	2	0.9 cm 0.2 cm	Screening ultrasound	.
6	Female	PTC-CMV	21	21	2	2 cm, 3 cm	Difficulties to swallow	desmoid tumors
7	Female	PTC	18	42	1	0.9 cm	Screening ultrasound	unspecified extrathyroid cancer
8	Male	PTC	20	44	2	0.8 cm and smaller	Screening ultrasound	desmoid tumors, adrenal adenoma
9	Female	PTC	16	62	1	0.4 cm	Screening ultrasound	ampullary cancer
10	Male	PTC	23	28	1	1.6 cm	Screening ultrasound	desmoid tumors
11	Female	PTC-FV	24	24	1	0.9 cm	Jugular vein thrombus	desmoid tumors
12	Male	PTC	44	45	2	0.6 cm 0.4 cm	Screening ultrasound	desmoid tumors

Additional file 4: **Figure S1.** ALK and KMT2D (MLL2) converge on CTNNB1. An evaluation of the potential role of the gene products from mutated genes in our cohort that are associated with negative outcomes was completed to potentially identify pathways that could be targeted for novel therapy in this young group. String®, a protein-protein network modeling website sponsored by the String Consortium and the Swiss Institute of Bioinformatics (SIB) [], was used to postulate how the gene mutations were connected in biochemical pathways that could potentially create the carcinogenic phenotype. In our cohort, ALK and KMT2D (MLL2) gene mutations were found to be associated with both ovarian and endometrial negative outcomes in patients with neuroendocrine histology. In the String program, the ALK and KMT2D proteins, while being involved in multiple pathways, appear to converge on CTNNB1, a key member of the Wnt pathway. The Wnt pathway is important in cell adhesion and maintenance of an appropriate cell cycle and has been found to be compromised in a variety of cancers. Additionally, dysregulation of the Wnt pathway has recently been implicated in maintenance of cancer stem cells, metastasis and immune control (Zhan T et al., Oncogene 2017; 36:1461–1473). The String algorithm also revealed that HSP90AA1 is also involved in this complex pathway. HSP90AA1 has been shown to be a prognostic indicator of both liver and breast cancers. () (DOCX 687 kb)