APC Transcription Studies and Molecular Diagnosis of Familial Adenomatous Polyposis

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Running Title: *APC* Transcription Studies in FAP

Conflict of Interest

There are no conflicts of interest to declare

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Abstract

Familial Adenomatous Polyposis (FAP) is characterised by the development of hundreds to thousands of colorectal adenomas and results from inherited or somatic mosaic variants in the *APC* gene. Index patients with suspected FAP are usually investigated by *APC* coding region sequence and dosage analysis in a clinical diagnostic setting. The identification of an *APC* variant which affects protein function enables predictive genetic testing to guide the management of family members. This report describes a 4-generation family with a phenotype consistent with FAP, but in which an *APC* variant had not been identified, despite testing. To explore this further, quantitative PCR (qPCR) was employed to assess *APC* transcription, demonstrating reduced levels of *APC* RNA. Next generation sequencing (NGS) identified the *APC* 5'UTR/ Exon 1 variant, c.-190 G>A, that had been reported previously in another FAP family with *APC* allelic imbalance. Quantitative RNA studies and DNA sequencing of the *APC* promoters/ Exon 1 may be useful diagnostically for patients with suspected FAP when coding region variants cannot be identified.

Key Words

- Colorectal adenomas
- Colorectal polyposis
- Familial adenomatous polyposis
- *APC*
- Quantitative Polymerase Chain Reaction (qPCR)

Introduction

Familial Adenomatous Polyposis (FAP), due to germline or somatic mosaic variants affecting *APC*, is the second most common cause of inherited colorectal cancer (CRC) after Lynch Syndrome. FAP affects approximately 1 in 8000 individuals (reviewed in ref 1; reviewed in ref 2; reviewed in ref 3). It is characterised by the development of hundreds to thousands of colorectal adenomas and, if untreated, will progress to CRC. In addition to FAP, there are several other, rarer syndromes which are characterised by multiple, though usually fewer, colorectal adenomas and an increased risk of CRC. These include *MUTYH*-Associated Polyposis (MAP) (ref 4), Polymerase-Proofreading Associated Polyposis (PPAP) (ref 5), *NTHL1*-Associated Polyposis (NAP) (ref 6) and *MSH3*-Associated Polyposis (ref 7).

The identification of causative variants in families with inherited polyposis syndromes is important for the prevention of CRC. Good clinical practice includes referral of patients with multiple colorectal adenomas for genetic counselling and consideration of diagnostic testing of *APC* and other polyposis genes. Well over 90% of patients with a phenotype of classical FAP have a germline *APC* variant affecting protein function identified through sequencing of coding exons and deletion/duplication analysis via multiplex ligation-dependent probe analysis (MLPA) (ref 8). Of those patients with an attenuated phenotype, with <100 adenomas, *APC*, *MUTYH* or other causative germline variants are detected in only 20-50% of cases (ref 8). The monogenic mechanisms potentially operating in the group who have no *APC* variant identified (NVI) include

promoter and other non-coding variants, somatic mosaicism, the involvement of other genes and epigenetic effects.

This paper describes a 4-generation family with a clinical diagnosis of FAP. Despite genetic diagnostic testing performed in several expert centres, the genetic basis for the disease had not been determined.

Methods

Subjects

A study at Cardiff University, 'Genetic Mechanisms in Colorectal Polyposis' (approved by the NHS Research Ethics Committee for Wales: REC 3, study 12/WA/0071) is currently investigating patients with at least 10 colorectal polyps who have no pathogenic *APC* variant identified (NVI) on genetic testing in a clinical diagnostic setting. All patients recruited to the study give written, informed consent.

One of the probands (Individual 2.1, Figure 1) participating in the study was a 44-year old female who had undergone a colectomy and proctectomy for clinical FAP. At least 11 other family members were also affected (Figure 1). The family history was provided by recruited family members but detailed clinical information on other family members was not available.

Quantitative PCR (qPCR)

APC transcript levels in leukocyte RNA were first determined within a healthy control cohort. RNA was prepared from venous blood samples from 40 normal controls, including 7 unaffected adult relatives of NVI patients and 33 healthy individuals without a personal or family history of polyposis or CRC recruited through a local study: Causes of Bowel Polyps: Recruitment of Healthy Controls (approved by Cardiff University School of Medicine Ethics Committee). RNA was converted to cDNA, which underwent qPCR using Taqman technology (details of experimental protocols are in Supplementary 1 and 2, available at the European Journal of Human Genetics webpage). cDNA from Individual 2.1 underwent qPCR using the same methods, along with a positive control with FAP due to a previously characterised *APC* promoter deletion that abrogated transcription, FAP1. Results were analysed using ThermoFisher Cloud software, and *APC* expression levels in Individual 2.1 and the FAP positive control were compared to the healthy cohort to give an Rq value.

Ultra-Deep Sequencing (UDS), Variant Calling and Validation in DNA

UDS across the whole genomic locus of *APC*, hg19 chr5:g.112042936-112186350, was undertaken in genomic DNA extracted from whole blood from Individual 2.1. Reference sequence NM_001127511.2 was used, and the first delimited exon, which is untranslated, was assigned as Exon 1.

Target sequence capture was undertaken using the Haloplex assay (Agilent). Sequencing was performed by the Wales Gene Park Genomics Facility

(http://www.walesgenepark.cardiff.ac.uk/next-generation-sequencing/) using the HiSeq (Illumina). Rare variants, present in </= 1% of the population, according to dbSNP data or The 1000 Genomes Project data, were analysed using CADD software (http://cadd.gs.washington.edu/home) and were assessed using the Integrative Genomics Viewer (IGV) (https://www.broadinstitute.org/igv/). Variants which had a CADD score >/= 15 were validated with Sanger sequencing (details in Supplementary 3, available at the European Journal of Human Genetics webpage).

Results

qPCR Studies

The *APC* Rq value for FAP1 was 0.48 and for Individual 2.1 it was 0.56 (Mean delta Ct = 8.238). An assessment for *APC* allelic imbalance in Individual 2.1 was attempted but homozygosity for the chosen SNPs in her genomic DNA precluded informativity of the analysis.

The qPCR results for all controls and NVI polyposis patients are in Supplementary 4, available at the European Journal of Human Genetics webpage. A further 3 NVI polyposis patients also had apparently reduced *APC* expression, but the cause in these patients was not identified despite *APC* ultradeep sequencing, karyotype analysis where possible and *APC* promoter methylation studies.

APC Capture and Ultra-Deep Sequencing (UDS)

The mean depth of coverage across the *APC* locus for Individual 2.1 was 2458 reads, with 97.7% of the target region covered at a minimum of 1x read and 73.4% at a

minimum of 1000x reads. The *APC* promoter/Exon1 variant c.-190 G>A (hg19 chr5:g.112043225 G>A) was identified in 2845/5546 (51%) reads and confirmed by Sanger sequencing. It had a CADD score of 22.4. The proband's father and one cousin, both of whom also had a clinical diagnosis of FAP, were subsequently recruited for investigation (Figure 1 Individuals 1.1 and 2.2). Both were found to carry the c.-190 G>A variant. qPCR studies gave Rq values of 0.56 for the father and 0.63 for the cousin. The variant has been submitted to the LOVD database (patient ID 00213111).

Discussion

Li *et al* (2016) (ref 9) previously reported the *APC* c.-190 G>A variant in another family with FAP and profuse fundic gland polyposis, in which 5 individuals were affected over 3 generations. The authors performed electromobility shift assays showing that the variant led to reduced protein binding, and that the protein likely to be affected was the transcription factor YY1. They demonstrated allelic imbalance of *APC* expression in carriers of the variant due to abrogation of transcription.

This is consistent with the observation that truncating *APC* germline mutations usually do not result in nonsense mediated decay (NMD) (ref 10, reviewed in ref 11) so when reduced *APC* expression is identified, it may be more likely to result from reduced transcription caused by promoter alterations rather than a 'missed' truncating variant.

In Li's paper, other specific germline point mutations in the *APC* promoter 1B region resulted in a phenotype of gastric polyposis. Individual 2.1 was found to have 'multiple

fundic gland polyps in the body and fundus' when she attended upper gastrointestinal endoscopic surveillance, but details of upper GI endoscopy could not be obtained for other family members.

The variant has been categorised as 'disease causing' (HGMD Accession CR165704) and predictive genetic testing is now being offered to relatives of Individual 2.1.

Our findings suggest that quantification of *APC* transcription may help to direct the search for an unusual underlying genetic mechanism in NVI patients with colorectal polyposis.

Other studies have also demonstrated the importance of analysing transcripts in the search for unusual underlying mechanisms in NVI polyposis patients. As early as 1993, Powell *et al* (ref 12) used an allele-specific expression assay to show that 3/11 *APC* NVI patients with clinical FAP had significantly reduced expression of one *APC* allele. In 1999 Laken *et al* (ref 13) used monoallelic mutation analysis (MAMA) to reveal that 7/9 *APC* NVI patients had reduced/ no expression from one of their *APC* alleles. More recently Yan *et al* (ref 14) identified a patient with colorectal tumours and reduced levels of the APC protein. This patient had the expected 50:50 *APC* allelic ratio in gDNA, but a 66:34 *APC* allelic ratio in cDNA from lymphoblastoid cells (ref 14). Early findings regarding *APC* AI have been supported by Castellsagué *et al* (ref 15). Of 23 *APC/ MUTYH* NVI polyposis families who were heterozygous for the SNP rs2229992, 2 were shown to exhibit *APC* AI. The AI in one family was suggested to result from

promoter variants (ref 15). In 2012 transcript analysis in a sample of 125 NVI polyposis patients found that 8% had a reproducible aberrant transcript pattern, the majority of which reflected insertions between two exons originating from exonised sequences deep within the corresponding intron (ref 8).

Considering *APC* qPCR studies, a rigorously determined Rq threshold would be required for diagnostic translation of transcription assays. With whole genome sequencing emerging as a realistic basis for genetic diagnosis it is likely that many more potentially regulatory non-coding variants will be identified in future. In this case, transcription studies and/or transcript analysis might form a second line test to provide evidence for or against their pathogenicity.

Conflict of interest

There are no conflicts of interest to declare.

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Figure legends

Figure 1 Family Pedigree. Proband is marked by an arrow (2.1)

Figure

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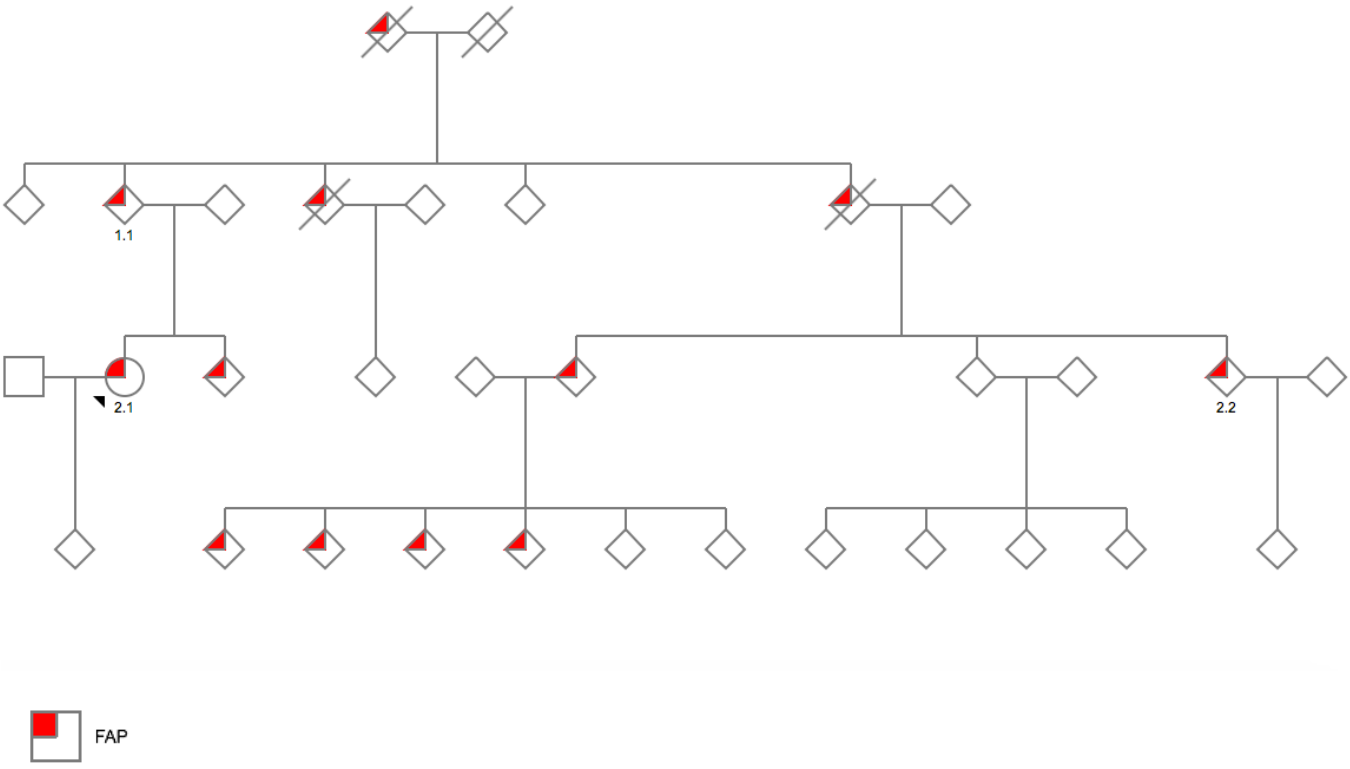

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