

1 *APC* Transcription Studies and  
2 Molecular Diagnosis of Familial  
3 Adenomatous Polyposis

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16 **Running Title:** *APC* Transcription Studies in FAP

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18 **Conflict of Interest**

19 There are no conflicts of interest to declare

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21 **Grants**

22 This work was supported by grants from The Pathological Society of Great Britain and  
23 Ireland and from Health and Care Research Wales to the Wales Gene Park and Wales  
24 Cancer Research Centre.

25

26 **Abstract**

27 Familial Adenomatous Polyposis (FAP) is characterised by the development of  
28 hundreds to thousands of colorectal adenomas and results from inherited or somatic  
29 mosaic variants in the *APC* gene. Index patients with suspected FAP are usually  
30 investigated by *APC* coding region sequence and dosage analysis in a clinical diagnostic  
31 setting. The identification of an *APC* variant which affects protein function enables  
32 predictive genetic testing to guide the management of family members. This report  
33 describes a 4-generation family with a phenotype consistent with FAP, but in which an  
34 *APC* variant had not been identified, despite testing. To explore this further,  
35 quantitative PCR (qPCR) was employed to assess *APC* transcription, demonstrating  
36 reduced levels of *APC* RNA. Next generation sequencing (NGS) identified the *APC*  
37 5'UTR/ Exon 1 variant, c.-190 G>A, that had been reported previously in another FAP  
38 family with *APC* allelic imbalance. Quantitative RNA studies and DNA sequencing of  
39 the *APC* promoters/ Exon 1 may be useful diagnostically for patients with suspected  
40 FAP when coding region variants cannot be identified.

41

42 **Key Words**

- 43 • Colorectal adenomas
- 44 • Colorectal polyposis
- 45 • Familial adenomatous polyposis
- 46 • *APC*
- 47 • Quantitative Polymerase Chain Reaction (qPCR)

48

49 **Introduction**

50 Familial Adenomatous Polyposis (FAP), due to germline or somatic mosaic variants  
51 affecting *APC*, is the second most common cause of inherited colorectal cancer (CRC)  
52 after Lynch Syndrome. FAP affects approximately 1 in 8000 individuals (reviewed in ref  
53 1; reviewed in ref 2; reviewed in ref 3). It is characterised by the development of  
54 hundreds to thousands of colorectal adenomas and, if untreated, will progress to CRC.  
55 In addition to FAP, there are several other, rarer syndromes which are characterised by  
56 multiple, though usually fewer, colorectal adenomas and an increased risk of CRC.  
57 These include *MUTYH*-Associated Polyposis (MAP) (ref 4), Polymerase-Proofreading  
58 Associated Polyposis (PPAP) (ref 5), *NTHL1*-Associated Polyposis (NAP) (ref 6) and  
59 *MSH3*-Associated Polyposis (ref 7).

60

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

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

73

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

77

78 **Methods**

79 **Subjects**

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

85 One of the probands (Individual 2.1, Figure 1) participating in the study was a 44-year  
86 old female who had undergone a colectomy and proctectomy for clinical FAP. At least  
87 11 other family members were also affected (Figure 1). The family history was  
88 provided by recruited family members but detailed clinical information on other family  
89 members was not available.

90

### 91 [Quantitative PCR \(qPCR\)](#)

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

105

### 106 [Ultra-Deep Sequencing \(UDS\), Variant Calling and Validation in DNA](#)

107 UDS across the whole genomic locus of *APC*, hg19 chr5:g.112042936-112186350, was  
108 undertaken in genomic DNA extracted from whole blood from Individual 2.1.  
109 Reference sequence NM\_001127511.2 was used, and the first delimited exon, which is  
110 untranslated, was assigned as Exon 1.  
111 Target sequence capture was undertaken using the Haloplex assay (Agilent).  
112 Sequencing was performed by the Wales Gene Park Genomics Facility

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

120

## 121 Results

### 122 qPCR Studies

123 The *APC* Rq value for FAP1 was 0.48 and for Individual 2.1 it was 0.56 (Mean delta Ct =  
124 8.238). An assessment for *APC* allelic imbalance in Individual 2.1 was attempted but  
125 homozygosity for the chosen SNPs in her genomic DNA precluded informativity of the  
126 analysis.

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

### 132 *APC* Capture and Ultra-Deep Sequencing (UDS)

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

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

142

#### 143 Discussion

144 Li *et al* (2016) (ref 9) previously reported the *APC* c.-190 G>A variant in another family  
145 with FAP and profuse fundic gland polyposis, in which 5 individuals were affected over  
146 3 generations. The authors performed electromobility shift assays showing that the  
147 variant led to reduced protein binding, and that the protein likely to be affected was  
148 the transcription factor YY1. They demonstrated allelic imbalance of *APC* expression in  
149 carriers of the variant due to abrogation of transcription.

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

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

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

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

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

166 Other studies have also demonstrated the importance of analysing transcripts in the  
167 search for unusual underlying mechanisms in NVI polyposis patients. As early as 1993,  
168 Powell *et al* (ref 12) used an allele-specific expression assay to show that 3/11 *APC* NVI  
169 patients with clinical FAP had significantly reduced expression of one *APC* allele. In  
170 1999 Laken *et al* (ref 13) used monoallelic mutation analysis (MAMA) to reveal that 7/9  
171 *APC* NVI patients had reduced/ no expression from one of their *APC* alleles. More  
172 recently Yan *et al* (ref 14) identified a patient with colorectal tumours and reduced  
173 levels of the *APC* protein. This patient had the expected 50:50 *APC* allelic ratio in  
174 gDNA, but a 66:34 *APC* allelic ratio in cDNA from lymphoblastoid cells (ref 14). Early  
175 findings regarding *APC* AI have been supported by Castellsagué *et al* (ref 15). Of 23  
176 *APC*/*MUTYH* NVI polyposis families who were heterozygous for the SNP rs2229992, 2  
177 were shown to exhibit *APC* AI. The AI in one family was suggested to result from

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

182

183

184 Considering *APC* qPCR studies, a rigorously determined Rq threshold would be

185 required for diagnostic translation of transcription assays. With whole genome

186 sequencing emerging as a realistic basis for genetic diagnosis it is likely that many

187 more potentially regulatory non-coding variants will be identified in future. In this

188 case, transcription studies and/or transcript analysis might form a second line test to

189 provide evidence for or against their pathogenicity.

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191 **Conflict of interest**

192 There are no conflicts of interest to declare.

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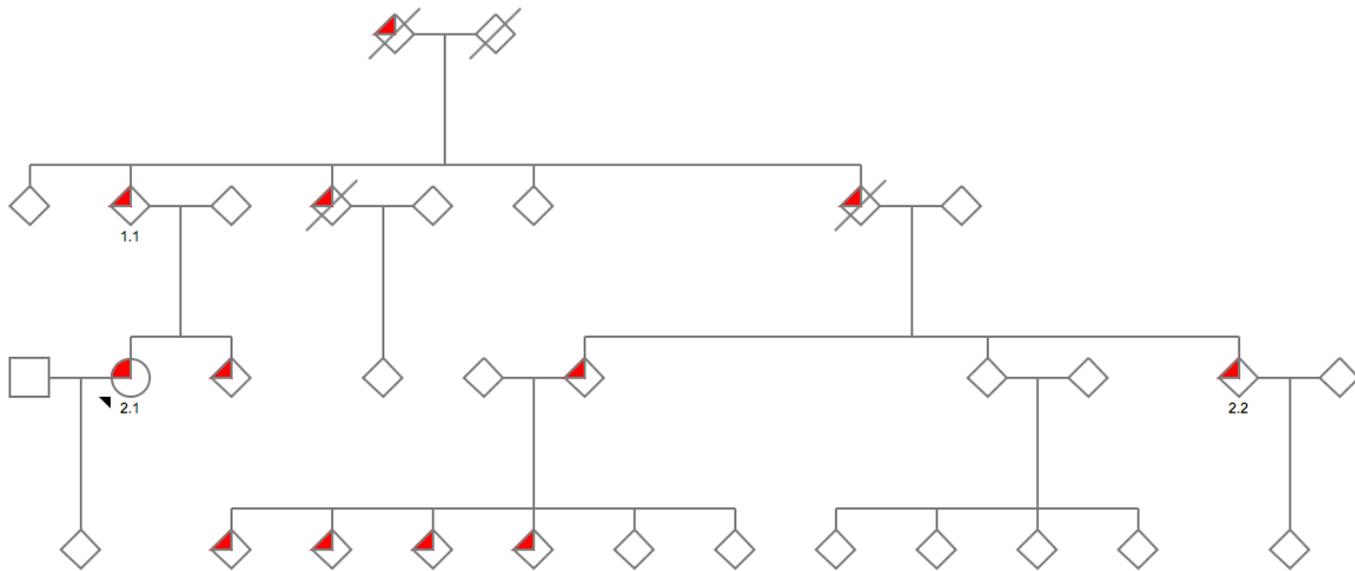
## 244 Figure legends

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

246

## 247 Figure

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