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ORIGINAL PAPER

Oncogenic *KRAS* is not necessary for Wnt signalling activation in APC-associated FAP adenomas

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Abstract

Recent studies have suggested that APC loss alone may be insufficient to promote aberrant Wnt/β-catenin signalling. Our aim was to comprehensively characterize Wnt signalling components in a set of APC-associated familial adenomatous polyposis (FAP) tumours. Sixty adenomas from six FAP patients with known pathogenic *APC* mutations were included. Somatic *APC* and *KRAS* mutations, β-catenin immunostaining, and qRT-PCR of *APC*, *MYC*, *AXIN2* and *SFRP1* were analysed. Array-comparative genomic hybridization (aCGH) was also assessed in 26 FAP adenomas and 24 paired adenoma–carcinoma samples. A somatic *APC* alteration was present in 15 adenomas (LOH in 11 and four point mutations). *KRAS* mutations were detected in 10% of the cases. *APC* mRNA was overexpressed in adenomas. *MYC* and *AXIN2* were also overexpressed, with significant intra-case heterogeneity. Increased cytoplasmic and/or nuclear β-catenin staining was seen in 94% and 80% of the adenomas. β-Catenin nuclear staining was strongly associated with *MYC* levels (*p* value 0.03) but not with *KRAS* mutations. Copy number aberrations were rare. However, the recurrent chromosome changes observed more frequently contained Wnt pathway genes (*p* value 0.012). Based on β-catenin staining and Wnt pathway target genes alterations the Wnt pathway appears to be constitutively activated in all APC-FAP tumours, with alterations occurring both upstream and downstream of APC. Wnt aberrations are present at both the DNA and the RNA level. Somatic profiling of APC-FAP tumours provides new insights into the role of APC in tumourigenesis.
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Introduction

Classical familial adenomatous polyposis (FAP) is most often caused by truncating germline mutations typically located in the central region of the *Adenomatous Polyposis Coli* (*APC*) tumour suppressor gene [1]. In some cases, missense mutations can also occur [2,3]. *APC* is also somatically mutated in sporadic colorectal cancer (CRC) at a high frequency. *APC* mutations can be detected in aberrant crypt foci, suggesting that the loss of APC function represents an initiating event in CRC [4]. Inactivation of both *APC* alleles is both necessary and sufficient to promote adenoma growth [5].

Molecular analyses of FAP-associated tumours have provided deep insights into tumourigenesis. Biallelic mutation of the *APC* gene is a hallmark of the colorectal, duodenal, and desmoid tumours that develop in FAP patients. The site of the 'first hit' in the *APC* tumour suppressor gene determines the type of the 'second hit'. Mutations near codon 1300 [codons 1285–1378; in the mutation cluster region (MCR)] [6] are associated with loss of heterozygosity (LOH), with no loss of genetic material [7]. More recently, putative 'third hits', mostly copy number gains or deletions, have been reported [8]. Combined profiling of mouse and human adenomas has allowed the identification of new direct and indirect target genes such as *BUB1, MAD2L1*, and *CD44*, which are associated with APC-driven tumour progression [9–14].

The APC protein plays an integral role in the Wnt signalling pathway, as it binds and down-regulates β-catenin [15] by the formation of a protein complex

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Table 1. Clinical characteristics of patients included in the study

ID	Sex	Family history	Age at operation (years)	No of adenomas	Extracolonic disease	CHRPE	Germline *APC* mutation
FAP1	F	Yes	35	300	No	No	c.1958+1G>A; r.[=, 1744_1958del]; p.?
FAP3	M	No	41	>100	No	No	c. [1958+3A>G; 1959G>A]; r.[=; 1744_1958del; 1959G>A]; p.?
FAP4	M	No	26	>1000	Yes	No	c.4175C>A; p.Ser1392X
FAP6	M	No	23	>100	Yes	No	c.4612_4613delGA; p.Glu1538IlefsX5
FAP8	F	Yes	20	139	No	No	c.1262_1263delinsAA; p.Trp412X
FAP9	F	Yes	33	800	No	No	c.3183_3187del; p.Gln1062X

F = female; M = male; CHRPE = congenital hypertrophy of the retinal pigment epithelium.

with AXIN and glycogen synthase kinase-3β (GSK3-β) [16]. Loss of functional APC, usually due to truncating mutations that remove the β-catenin regulatory domain, leads to nuclear accumulation of β-catenin [15], where it acts as a TCF4 transactivator. TCF4 transcriptionally targets pro-proliferative genes such as *MYC* and *cyclin D1*, which are key effectors of this pathway [17,18]. The activated β-catenin/TCF4 complex imposes a progenitor-like phenotype in colorectal cells by regulating MYC and p21 activities [19]. Wnt signalling is autoregulated at many levels. The expression of a variety of positive and negative regulators of the pathway, such as *FRIZZLEDs, LRP* and *HSPG, AXIN2* and *TCF/Lef*, is controlled by the β-catenin/TCF complex. Recent studies have suggested that APC loss alone may be insufficient to promote aberrant Wnt/β-catenin signalling and that APC and KRAS play distinct roles in the control of stability and nuclear accumulation of β-catenin [20].

The aim of this study was to gain further insight into the role of APC and the Wnt signalling members in APC-FAP adenomas. We collected FAP adenomas and corresponding mucosae and CRC samples, and screened the *APC* gene as well as *KRAS*. We also studied the expression and DNA copy number changes of key members of the pathway both downstream and upstream of APC.

Materials and methods

FAP colorectal adenomas

Fresh samples from colectomy specimens of six FAP patients who harboured *APC* pathogenic germline mutations were collected before fixation and cryopreserved (Table 1). Ten adenomas representative of the five areas of the colon and rectum (caecum, ascendant, transverse, descendant colon, and rectum) and paired normal mucosa from each patient were analysed. Adenomas were classified according to size as small, <10 mm; medium, 10–20 mm; or large, >20 mm (Supporting information, Supplementary Table 1). A set of ten paired normal mucosa/adenoma/carcinoma samples was also analysed. In all cases, at least 70% of tumour cell content was present. Written informed consent was obtained from all patients participating in the study. The study protocol was approved by the Ethics Committee of the Hospital Universitari de Bellvitge.

APC and *KRAS* mutations

APC and *KRAS* mutations were analysed by sequencing. Genomic DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA, USA). *APC* primers were designed to amplify 838 nt that contained codons 1203–1482 of the gene and for *KRAS* a 241 nt fragment containing codons 12 and 13 (Supporting information, Supplementary Table 2). The PCR products were then purified with the Jetquik PCR purification kit (Genomed, Kent, UK) and directly sequenced using ABI PRISM 37.30 (Applied Biosystems Inc, Foster City, CA, USA).

APC LOH analysis

LOH at *APC* was analysed using three microsatellite markers on chromosome 5q (D5S82, D5S346, and D5S299) (Supporting information, Supplementary Table 2). Primers are available upon request. Products were detected using ABI PRISM 37.30 (Applied Biosystems Inc). In informative cases, the values given for the peak area of the two alleles in the paired normal and tumour samples were used to define allele loss as described by Cawkwell *et al* [21]. A ratio of less than or equal to 0.30 was assigned to be indicative of loss on the basis that some tumours in the series contained up to 30% of normal cells contamination.

APC allele-specific expression (ASE) analysis

ASE at *APC* was analysed using the rs2229992 SNP on cDNA (Supporting information, Supplementary Table 2). To specifically amplify cDNA, we used an exon 11 forward primer (5′-GGGACTACAGGCCATTGCA-3′) and a reverse primer targeting the exon 11–12 junction (5′-ATAGAGCATAGCGTAGCCTTGTTG-3′). PCR products were purified using illustraTM GFXTM PCR DNA and the Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK.). For the single nucleotide primer extension reaction, primer extension was carried out with the SNaPshot Multiplex Kit (Applied Biosystems) with 5′-TATTGCAAGTGGACTGTGAAATGTA-3′ according to the manufacturer's instructions.

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Table 2. *APC* alterations in FAP adenomas

	Somatic				*APC* expression levels	
ID	Point mutation*	LOH	aCGH loss	Total	Normal mucosa†	Adenomas with underexpression‡
FAP1	0	5	4 (3)§	6/10 (60%)	1.676	9/10
FAP3	1	0	1	2/10 (20%)	0.977	3/10
FAP4	0	3	0	3/10 (30%)	1.576	4/10
FAP6	0	2	0	2/10 (20%)	1.932	9/10
FAP8	1	0	0	1/10 (10%)	0.634	0/10
FAP9	2	1	0	3/10 (30%)	0.775	4/10

* The somatic point mutations detected were as follows: FAP3 AD2: Q1338X C>T; FAP8 AD7: E1397X G>T; FAP9 AD1: S1356XC>G; AD4: E1309X G>T.
† Basal expression refers to $\log_2$ values of macroscopically normal mucosa versus a pool of normal colonic mucosa from sporadic colorectal cancer cases.
‡ Underexpression in adenomas is defined as $\log_2$ values <0 when compared with corresponding normal mucosa of the same FAP patient.
§ In parentheses, cases with concomitant LOH and loss of genetic material as assessed by aCGH.

SNaPshot reaction products were treated with 1 U of shrimp alkaline phosphatase (usb) for 60 min at 37 °C and then for 15 min at 75 °C. Products were run in an ABI Prism 3130 DNA sequencer and analysed by GeneMapper v4.0 (Applied Biosystems).

ASE was measured using peak intensities in heterozygous samples. Allelic frequencies were calculated as freq C = C/(C + kT) or freq T = T/(T + k'C), where k and k' are constants given by the mean of the C/T(k) and T/C(k') proportions in control samples. ASE values are expressed as the proportion of frequencies of both alleles (freq C/freq T) and are normalized using two normal mucosae from sporadic patients. Three independent replicates of all experiments were obtained and in every experiment a set of controls was included. A Mann–Whitney test was used to evaluate ASE differences among groups.

β-Catenin immunostaining

Five-micrometre sections of biopsy specimens were treated with 3% formaldehyde. After blocking with 3% hydrogen peroxide, the sections were incubated with the anti-β-catenin monoclonal antibody (BD Biosciences, San Jose, CA, USA) diluted 1 : 90 in PBS, followed by washing. Sections were incubated with the anti-mouse EnVision HRP System (Dako, Glostrup, Denmark), followed by washing. Results were independently analysed by two pathologists who assessed the localization and the level of β-catenin expression. β-Catenin staining was graded as none, weak, moderate, or strong (−, +, ++, +++) for both cytoplasmic and nuclear.

Gene expression analysis

Total RNA was isolated using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). One microgram of RNA was reverse-transcribed into cDNA using pdN6 primers and MMLV reverse transcriptase (Invitrogen). Subsequent real-time PCR reactions were performed in duplicate in the LightCycler® 2.0 System (Roche Diagnostics, Mannheim, Germany) using the SYBR Green detection methodology. Primers were designed to specifically amplify *MYC, SFRP1*, and *AXIN2* mRNA as they were placed in different exons of the genes (Supporting information, Supplementary Table 2). *APC* primers targeted exons 2 and 3, allowing the simultaneous analyses of all transcripts. β-*2-microglobulin* was used as an internal control for normalization. Threshold cycle data were analysed using the following formula

$$\text{ratio} = \left(\left(E_{\text{target}}\right)^{\Delta CP_{\text{target}}(\text{control}-\text{sample})}\right) / \left(\left(E_{\text{ref}}\right)^{\Delta CP\text{ref}(\text{control}-\text{sample})}\right)$$

[22] to quantify the level of gene expression changes. Expression levels were $\log_2$ ratios. The t-test and Wilcoxon test were applied to $\log_2$ ratios to evaluate their significance. The $\log_2$ ratios also allowed indirect comparisons between sporadic and FAP mucosae and tumours.

Array CGH analysis

DNA was isolated as previously described and quantified. DNA labelling was performed using the BioPrime DNA labelling kit reagents (Invitrogen) and according to protocols described elsewhere [23]. Labelled DNAs were hybridized to customized oligonucleotide microarrays containing 30 000 60-mer oligo probes assessing 449 chromosomal regions [23]. Fluorescence ratios of scanned images of arrays were obtained using BlueFuse version 3.2 (BlueGnome).

Array CGH data consist of the log ratios of normalized intensities, indexed by the physical location of the probes on the genome. Data were processed using the statistical package snapCGH (R package version 1.10.0) of Bioconductor [24] in the R software (http://www.R-project.org) and initially filtered for low-quality probes based on quality standard values. Each microarray was normalized focusing the median intensity in 0. Values were log-transformed and then the complete set of microarrays was normalized using the quantiles method. Default parameters of snapCGH were used for the segmentation process using the GLAD method [25,26] based on the adaptive weights smoothing (AWS) procedure. Each chromosome of each sample was processed separately and altered regions were compiled from different arrays.

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Array CGH raw data may be found in the Supporting information.

Results

APC somatic alterations in FAP adenomas

A somatic *APC* alteration was present in 15 of 60 (25%) adenomas analysed. LOH was detected in 11 out of 60 adenomas (18%) (Figure 1A and Table 2). In agreement with previous studies [25,26], concomitant loss of genetic material associated with LOH was observed in three cases only. Mutational analysis of somatic alterations in the *APC* gene was directed to the MCR. Only four somatic mutations were detected in the 60 adenomas studied (6%) (Figure 1B) and these were all nonsense mutations near to codon 1300, one of them being a C → T transition and the remaining being transversions. In two adenomas, two somatic alterations were detected.

APC mRNA overexpression is present in FAP adenomas

APC levels of each FAP adenoma were compared with their corresponding mucosa. Adenomas from all FAP patients presented increased *APC* expression levels (three-fold average) (Figure 1C). These changes were not observed in sporadic samples (adenomas and carcinomas) (Figure 1C). Macroscopically normal mucosa from FAP patients showed a 2.4-fold increase when compared with a pool of sporadic normal mucosae, indicating that abnormal overexpression is a very early event in APC-driven tumourigenesis. Altogether, *APC* RNA expression levels appeared elevated in FAP adenomas compared with sporadic adenomas (p value 0.0004, t-test).

Allele-specific expression (ASE) was explored in FAP3 tumours since it turned out to be heterozygous for rs2229992. The range for normal ASE (0.8–1.2) was established using colorectal normal mucosae. While FAP mucosa showed balanced expression of both alleles (0.826), ASE imbalance was present in four (values for individual adenomas: 0.691, 0.589, 0.561, and 0.349) of the five FAP adenomas analysed, suggesting that expression of the mutated allele can be selected for during tumour progression.

Wnt pathway is activated in adenomas and changes in expression occur upstream and downstream of APC

In FAP adenomas, some type of cytoplasmic β-catenin accumulation was present in 56 of 59 (94%) cases (Table 3). Nuclear accumulation was observed in 46 of 59 (80%) FAP adenomas but in none of the normal mucosae. Intensities of cytoplasmic and nuclear staining were positively correlated (p value 0.016; Fisher's exact test) (Figure 2 and Supporting information, Supplementary Table 1). The intensity of nuclear β-catenin staining correlated with adenoma size. Sixteen of 45 (35%) small adenomas (less than 1 cm) showed moderate to strong nuclear immunostaining (++ or +++), while this percentage increased to 78% (11 of 14) for adenomas larger than 1 cm ($p = 0.006$). Ten of the 15 cases (66%) showing biallelic *APC* inactivation showed either moderate ($n = 7$) or strong ($n = 3$) nuclear staining. There was no evidence of heterogeneity in this set of samples. Immunohistochemistry of sporadic samples showed nuclear staining in four of four adenomas and in eight of ten carcinomas.

To extend our studies to other Wnt-related genes, we examined the 60 matched sets of FAP adenomas together with ten sporadic adenomas and ten sporadic carcinomas for expression of CMYC, AXIN2, and SFRP1 (Figure 3). This analysis revealed up-regulation of *CMYC* in both sporadic (4.34-fold) and FAP tumours (5.82-fold). A statistically significant correlation was observed between β-catenin nuclear staining and elevated *CMYC* levels (p value 0.03; Kruskal–Wallis test). Furthermore, *AXIN2* (also known as conductin) was overexpressed in all the analysed adenomas (11.2-fold). *CMYC* and *AXIN2* expression levels correlated highly with those of *APC* (p value 0.02 and <0.0001, respectively; Pearson correlation test). In contrast, *SFRP1* was consistently down-regulated or even undetectable in FAP (19.5-fold) and sporadic tumours (45-fold). Moreover, *MYC* and *SFRP1* had altered expression levels not only in tumoural samples, but also in macroscopically normal mucosa (data not shown).

KRAS mutations are rare and are not associated with β-catenin nuclear accumulation

Six out of 60 (10%) FAP adenomas were carriers of a *KRAS* codon 12 mutation (Table 3 and Supporting information, Supplementary Table 1). The most frequent change (G-to-A mutation in the second position of codon 12) was identified in two of the adenomas with *KRAS* mutations. No correlation was observed between the presence of the mutation and β-catenin nuclear accumulation (Table 3).

Recurrent chromosome changes more commonly contain Wnt pathway genes

aCGH was performed in 29 of the 60 FAP adenomas and in 24 paired adenoma–carcinoma samples (Figure 4). We explored whether Wnt pathway components and their targets ($n = 130$) were overrepresented in areas with genomic losses or gains. FAP and sporadic samples were jointly analysed. Wnt genes were overrepresented in those areas showing copy number variation ($p = 0.01$; $p = 0.01$, Fisher's exact test and Pearson's chi-squared test, respectively). The Wnt genes present in most frequently gained regions were *GJB6* (gap junction protein, beta 6), *FGF9* (fibroblast growth factor 9), *TNFRSF19* (tumour necrosis factor receptor superfamily, member 19), *POSTN* (periostin), *TNFSF11* [tumour necrosis factor (ligand)

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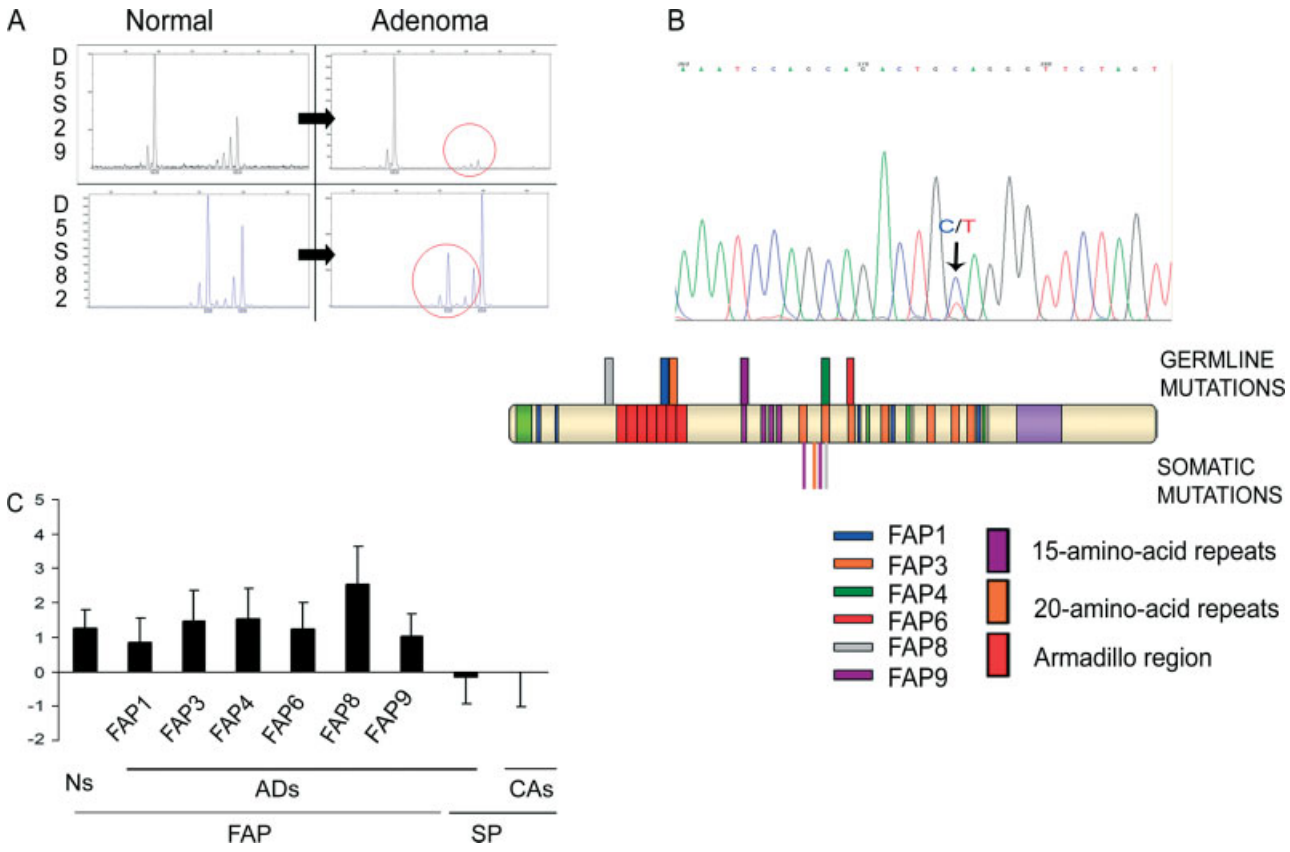

Figure 1. *APC* somatic alterations in FAP adenomas. (A) LOH was assessed in the *APC* gene by analysing three different markers. Representative example of complete loss of one *APC* allele (upper panel) and partial loss of another marker (lower panel). (B) Germline and somatic mutations in the *APC* gene. Schematic representation of the gene, where germline mutations are represented as wide bars and somatic mutations are represented as narrow bars. The MRC (mutation cluster region) region of the gene was screened for mutations in ten adenomas from each of the six FAP patients with known germline mutation. (C) *APC* relative mRNA levels in FAP and sporadic CRC samples. *APC* mRNA levels were assessed by means of quantitative PCR. FAP cases (mean value of all samples) were compared with a pool of ten sporadic CRC normal mucosae. The last two bars correspond to the mean values of the ten adenomas and carcinomas compared with their corresponding normal mucosa with minor changes in *APC* RNA expression.

superfamily, member 11], *JAG1* (jagged 1), *NKX2-2* (NK2 homeobox 2), *WISP2* (WNT1 inducible signalling pathway protein 2), *MMP9* (matrix metalloproteinase 9), and *SALL4* (sal-like 4). The Wnt genes found in frequently lost regions were *SMAD4* (SMAD family member 4) and *TCF4* (transcription factor 4).

Discussion

Genetic and epigenetic aberrations in several components of the Wnt signalling pathway have been found, at a high frequency, in colorectal cancers. In this study, we have gained insight into the scope and degree of Wnt pathway activation in APC-driven colorectal tumourigenesis at multiple levels with particular attention to the mRNA expression levels of relevant targets, β-catenin immunostaining, correlation with *KRAS* mutations, and the presence of copy number alterations.

A detectable second hit in the *APC* gene was found in 25% of APC-FAP adenomas, with LOH being more frequently found than point mutations. Other reports have described a similar rate of LOH (21–22%) [7,27], but our rate of somatic mutations in *APC* is lower. The relatively low prevalence of second mutations found may be related to the fact that only the MCR of the gene was analysed, leaving aside a high proportion of the coding region and all adjacent areas. As has been previously reported [7], LOH in FAP adenomas is not associated with loss of genetic material, further indicating that somatic recombination underlies it.

Consistent *APC* mRNA overexpression was observed in FAP samples, either morphologically normal or adenoma tissue. Our RT-PCR assay targets a common region shared by all *APC* isoforms, thus providing an overall assessment of all *APC* transcript levels. In contrast, diminished germline dosage of the APC alleles at both the DNA and the RNA level has been associated with the development of FAP [28–32]. However, and in line with our finding, Venesio *et al* [33] found increased germline expression of an *APC* mRNA isoform in AFAP patients. This observation and our ASE results could point to allele-specific expression of the aberrant and inactive form of APC. Further studies are required to explore whether the normal or the mutant allele is overexpressed. Regardless of whether RNA levels are up-regulated or down-regulated, subtle changes must tend to select a modest, maybe optimal,

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Table 3. *KRAS* and β-catenin status in FAP adenomas

Sample	*KRAS* somatic mutation	β-Catenin		*APC* biallelic mutation	FAP adenoma size
		Cytoplasm	Nucleus		
FAP4 AD5	–	++	+++	LOH	Large
FAP4 AD10	–	++	+++	LOH	Medium
FAP4 AD7	–	++	+++	–	Medium
FAP4 AD8	–	+	+++	LOH	Small
FAP4 AD9	–	++	+++	–	Small
FAP8 AD9	–	++	+++	–	Small
FAP8 AD8	–	++	+++	–	Small
FAP1 AD4	–	++	++	LOH	Large
FAP1 AD2	G12A G>C	+	++	LOH	Large
FAP1 AD5	–	+	++	LOH	Large
FAP1 AD7	–	++	++	LOH	Large
FAP1 AD3	–	+	++	–	Small
FAP1 AD6	–	++	++	LOH	Large
FAP1 AD1	–	++	++	–	Small
FAP3 AD5	–	+++	++	–	Medium
FAP3 AD6	–	++	++	–	Large
FAP4 AD4	–	++	++	–	Large
FAP4 AD3	–	+	++	–	Small
FAP6 AD6	–	+	++	–	Small
FAP6 AD4	–	++	++	–	Small
FAP6 AD3	–	++	++	–	Small
FAP6 AD2	–	+++	++	–	Small
FAP8 AD5	–	+	++	–	Small
FAP8 AD10	–	+	++	–	Small
FAP8 AD7	–	+	++	E1397X	Small
FAP8 AD6	–	+	++	–	Small
FAP9 AD3	–	+++	++	LOH	Small
FAP1 AD8	–	++	+/–	–	Small
FAP1 AD9	–	++	+/–	–	Small
FAP3 AD7	G12D G>A	++	+	–	Small
FAP3 AD8	–	++	+	–	Medium
FAP3 AD9	–	++	+	–	Small
FAP3 AD10	–	++	+	–	Small
FAP4 AD1	–	++	+	–	Small
FAP4 AD2	–	+	+	–	Small
FAP6 AD7	–	++	+	LOH	Small
FAP6 AD1	–	++	+	–	Small
FAP6 AD5	–	+	+	–	Small
FAP6 AD8	–	++	+	LOH	Small
FAP6 AD9	–	++	+	–	Small
FAP6 AD10	–	+	+	–	Small
FAP8 AD4	–	+	+	–	Small
FAP8 AD3	G12D G>A	++	+	–	Small
FAP9 AD2	–	++	+	–	Small
FAP3 AD3	G12S G>A	++	–/+	–	Small
FAP3 AD2	–	+	–/+	Q1338X	Small
FAP1 AD10	–	+	–	–	Small
FAP3 AD1	G12C G>T	++	–	–	Large
FAP3 AD4	–	–	–	–	Medium
FAP8 AD2	–	–	–	–	Small
FAP8 AD1	–	+	–	–	Small
FAP9 AD9	–	++	–	–	Small
FAP9 AD10	–	++	–	–	Small
FAP9 AD8	–	–	–	–	Small
FAP9 AD6	–	+	–	–	Small
FAP9 AD7	G12A G>C	+	–	–	Small
FAP9 AD4	–	+	–	–	Small
FAP9 AD1	–	+	–	S1356X	Small
FAP9 AD5	–	–	–	E1309X	Small
FAP4 AD6	–			–	Large

– = no mutation/no staining; + = weak staining; ++ = moderate staining; +++ = strong staining.

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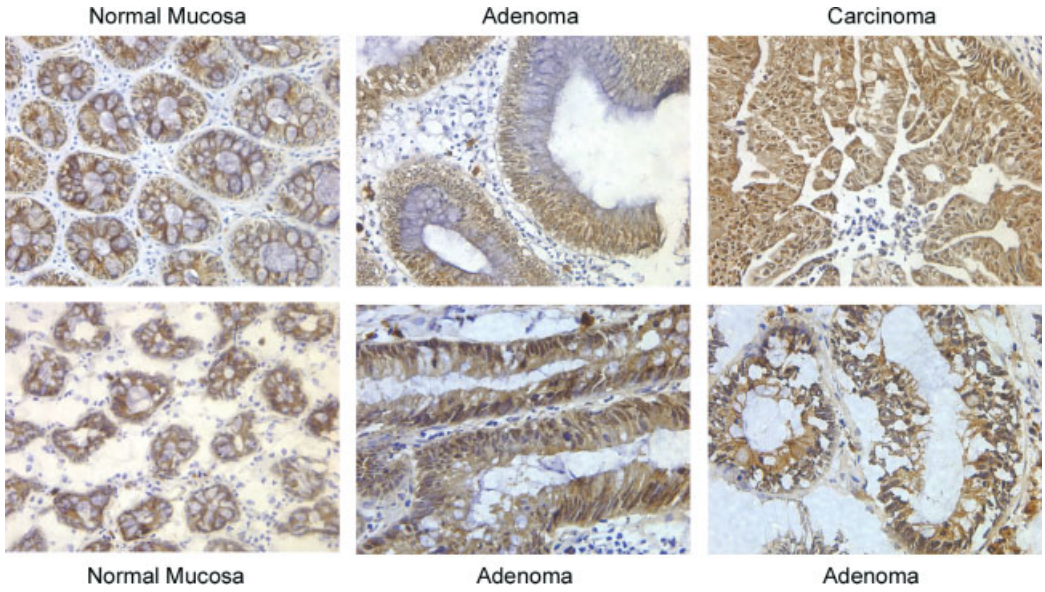

Figure 2. β-Catenin translocates to the nucleus in colorectal tumour samples. β-Catenin immunostaining of normal mucosa, an adenoma, and a carcinoma of sporadic colorectal cancer patients representative of all samples (upper panel). β-Catenin immunostaining of normal mucosa and two different adenomas of FAP patients representative of all samples (lower panel). β-Catenin is restricted to the cytoplasm in normal samples, whereas it accumulates in the cytoplasm and translocates to the nucleus in tumour samples.

APC expression level for FAP adenoma development. Our observations also indicate that this mRNA deregulation is not present in sporadic CRC samples, suggesting a different mechanism of Wnt activation and reinforcing the role of the first *APC* hit in FAP that not only influences the molecular nature of the second hit, but might also influence the level of *APC* mRNA expression.

Nuclear β-catenin immunostaining has been considered a surrogate of Wnt pathway activation that would occur upon homozygous loss of APC. Previous studies assessing nuclear β-catenin in FAP adenomas yielded controversial findings showing both positive [34,35] and negative results [20,36–38]. Discrepancies have been attributed to the technical challenges associated with β-catenin staining. Using an immunohistochemical technique that yielded consistent results both in fresh-frozen and in paraffin-embedded tissues, we detected some degree of nuclear immunostaining in 80% of the fresh-frozen FAP adenomas analysed. In a recent report, Phelps *et al* combined zebrafish, *in vitro* studies, and tumour analyses to conclude that loss of APC alone stabilizes the levels of cytoplasmic β-catenin but this stabilization is insufficient for causing β-catenin nuclear accumulation, which requires the activities of KRAS and RAF1 [20]. Our results not only confirm the APC-associated cytoplasmic accumulation of β-catenin, but also show that biallelic *APC* inactivation is strongly associated with moderate or strong nuclear staining, supporting the idea that APC total inactivation is driving and increasing Wnt signalling activation. In line with previous reports [39], a low prevalence of *KRAS* mutations was found. Importantly, no apparent relationship was observed with β-catenin nuclear accumulation. Thus, our results conflict with those of Phelps *et al*, showing that FAP adenomas with mono- or bi-allelic *APC* inactivation and without *KRAS* mutations are capable of inducing β-catenin nuclear translocation. While several animal models have demonstrated that *KRAS* mutation enhances the APC inactivation effect [40–49], the involvement of *KRAS* in β-catenin nuclear localization remains to be elucidated.

The Wnt pathway regulates development and cellular homeostasis and is well conserved through evolution. If early pathway activation is present, regulation of other members of the signalling pathway should occur. *MYC* was first described as a target of the Wnt pathway [50] and has been identified as a key effector of the β-catenin transcriptional programme [51]. *MYC* levels were up-regulated in the majority of adenomas and carcinomas, and correlated with β-catenin nuclear immunostaining. *AXIN2* was also overexpressed in FAP adenomas, in line with previous reports [52,53]. *AXIN2* overexpression can be used as a surrogate of Wnt signalling activation since it serves as a negative feedback loop for the Wnt signalling pathway [54]. Although *AXIN2* overexpression can down-regulate β-catenin in human tumour cell lines [54,55], a destruction complex with an inactive APC component is apparently not capable of eliminating β-catenin accumulation in FAP adenomas.

In agreement with previous reports [52,56], *SFRP1* was consistently down-regulated or even undetectable in FAP and sporadic tumours. *SFRP1* is a member of the SFRP family of proteins that act as inhibitors of Wnt signalling by preventing binding to its receptor. In normal Wnt signalling, *SFRP1* levels are regulated by β-catenin/TCF4 and promoter methylation is suggested by some authors as the mechanism by which *SFRP* transcription is inactivated [52,57]. In APC-driven tumourigenesis, *SFRP1* down-regulation is a very early

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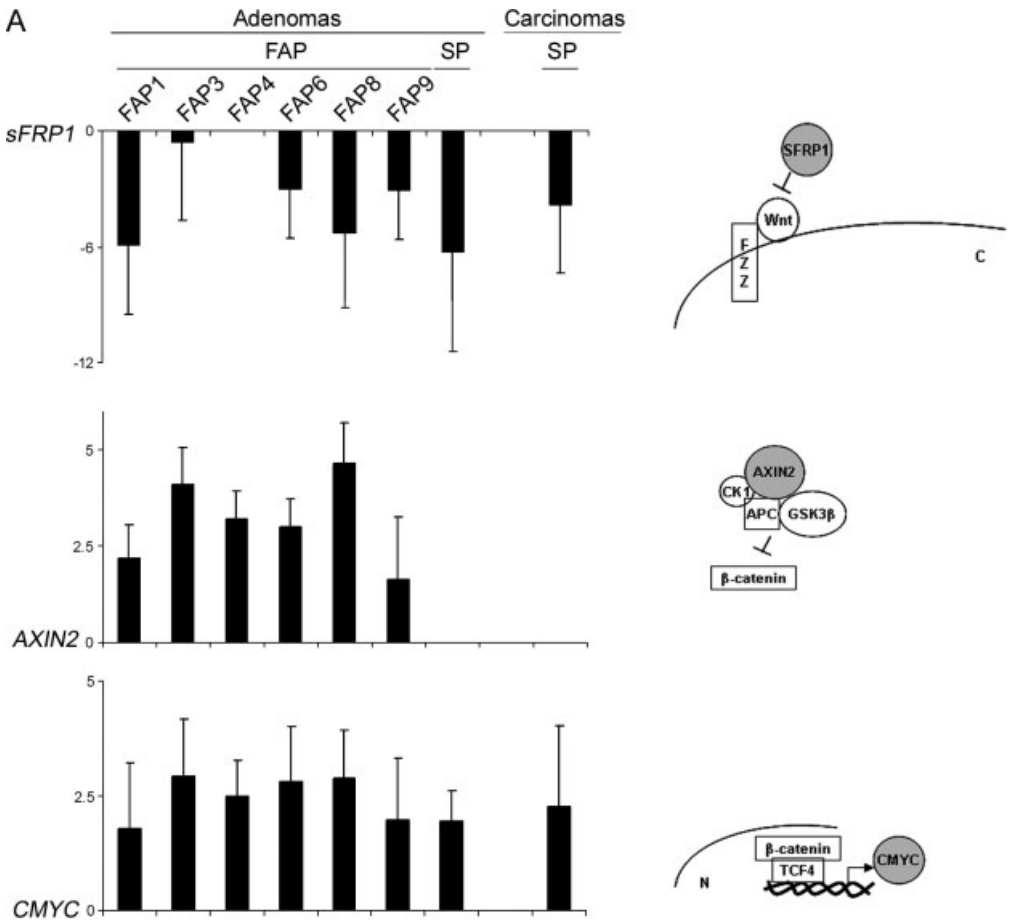

B

	C-MYC		sFRP1		AXIN2	
	log ratio	*p*-value	log ratio	*p*-value	log ratio	*p*-value
PCF1	1.78	0.019*	-24.16	0.002**	2.18	0.002**
PCF3	2.92	0.002**	-18.85	0.064	4.11	0.002**
PCF4	2.48	0.002**	ND	ND	3.21	0.002**
PCF6	2.82	0.002**	-27.39	0.002**	2.99	0.002**
PCF8	2.87	0.002**	-17.46	0.006**	4.65	0.002**
PCF9	1.98	0.002**	-3.06	0.084	1.62	0.013*

ND: non-detectable

Figure 3. Altered expression levels of Wnt signalling members in colorectal tumour samples. (A) Relative RNA expression levels of *SFRP1*, *AXIN2*, and *CMYC* expressed as $\log_2$ ratios (0 means no change). Mean values of the ten adenomas from the six FAP patients and mean values of ten sporadic (SP) colorectal cancer adenomas and carcinomas compared with their corresponding normal mucosa. Schematic representation of the protein function and location. SFRP1 binds to Frizzled (FZZ) receptors and prevents Wnt binding, and thus inhibits the pathway. AXIN2 is part of the β-catenin destruction complex and promotes β-catenin degradation by the proteasome. MYC is a target of the pathway and it promotes cell proliferation. (B) Log_2 ratio expression and *p* value for the three genes. $^{*}p < 0.05$; $^{**}p < 0.01$.

event that may promote further deregulation of the Wnt pathway.

The abnormal expression of *MYC* and *SFRP1* in the macroscopically normal mucosa in patients with FAP provides us with indirect evidence that the Wnt pathway is already activated in the very early stages of tumourigenesis. Previously, we have shown that overexpression of mitotic checkpoint proteins is present in both adenomas and normal mucosa from FAP patients [9]. Overall, our results point to an evident functional impact of a single *APC* mutated allele and our data are consistent with results obtained by Yeung *et al* using protein analysis techniques in normal colon crypts from FAP patients [58].

When we expanded our Wnt signalling pathway analysis to the DNA level, the combined analysis of FAP adenomas, sporadic adenomas, and carcinomas led to the observation that Wnt pathway components and target genes are overrepresented in areas with losses and gains, supporting the third-hit hypothesis where Wnt signalling modulation could be secondary to copy number changes in more advanced stages of tumour

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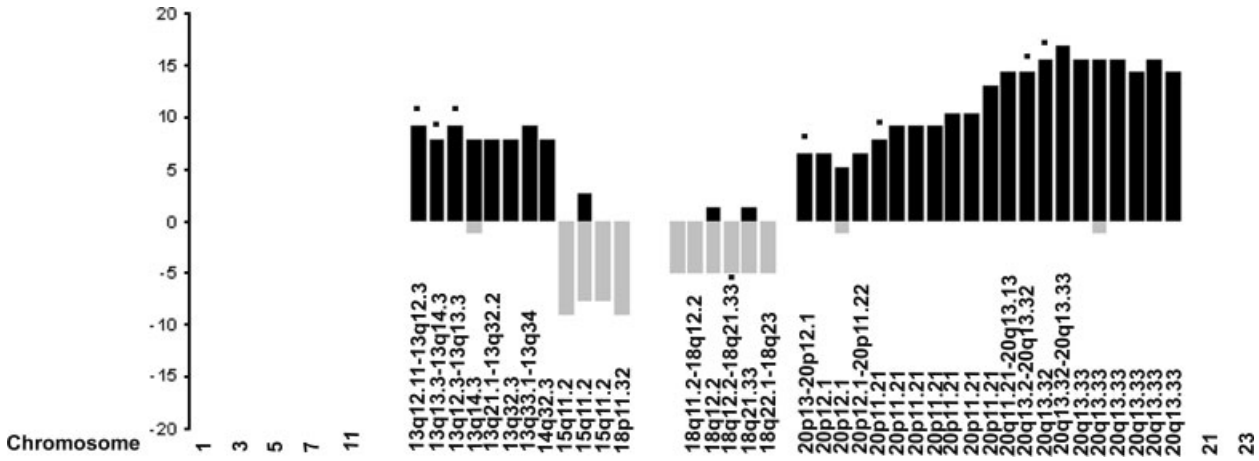

Figure 4. Array CGH data of colorectal tumours. Array CGH data are represented as a bar plot, where gains are shown in black and losses in grey. The *Y* axis represents the percentage of samples that harbour the change in copy number. Regions containing Wnt genes are marked with a dot.

progression [8]. Some of these Wnt genes, such as *JAG1*, a key member of the Notch signalling pathway [59], and *POSTN* [60], have been previously described to be altered in CRC. Our aCGH data further extend published results in APC- and MYH-FAP adenomas [61,62] and show that adenomas harbour a low level of genetic instability.

Taken together, our results suggest that monoallelic *APC* mutation might be sufficient for deregulation of the expression of *APC* and other key members of the Wnt pathway. While *APC* biallelic inactivation is associated with β-catenin nuclear localization, *KRAS* mutations do not appear to be necessary for this translocation. The data presented here further elucidate the mechanisms of APC-driven tumourigenesis. Early and universal transcriptional activation of the Wnt signalling pathway is evident at the RNA level, whilst further activation may occur later as genomic instability supervenes and copy number variations then arise. We have identified novel loci of genomic instability that may direct further investigation of potentially novel genes relevant to colorectal cancer genes.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Table S1. Summary of the results obtained with FAP and CRC samples.

Table S2. Primer sequences used for mutation detection, LOH detection, and quantitative PCR analysis.

Supplementary array data. aCGH raw data for sporadic colorectal cancer samples marked as AD for adenomas and CA for carcinomas.

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