**APC Splicing Mutations Leading to In-Frame Exon 12 or Exon 13 Skipping Are Rare Events in FAP Pathogenesis and Define the Clinical Outcome**

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**Abstract:** Familial adenomatous polyposis (FAP) is caused by germline mutations in the tumor suppressor gene APC. To date, nearly 2000 APC mutations have been described in FAP, most of which are predicted to result in truncated protein products. Mutations leading to aberrant APC splicing have rarely been reported. Here, we characterized a novel germline heterozygous splice donor site mutation in APC exon 12 (NM_000038.5: c.1621_1626+7del) leading to exon 12 skipping in an Italian family with the attenuated FAP (AFAP) phenotype. Moreover, we performed a literature meta-analysis of APC splicing mutations. We found that 119 unique APC splicing mutations, including the one described here, have been reported in FAP patients, 69 of which have been characterized at the mRNA level. Among these, only a small proportion (9/69) results in an in-frame protein, with four mutations causing skipping of exon 12 or 13 with loss of armadillo repeat 2 (ARM2) and 3 (ARM3), and five mutations leading to skipping of exon 5, 7, 8, or (partially) 9 with loss of regions not encompassing known functional domains. The APC splicing mutations causing skipping of exon 12 or 13 considered in this study cluster with the AFAP phenotype and reveal a potential molecular mechanism of pathogenesis in FAP disease.

**Keywords:** familial adenomatous polyposis; APC; splicing; exon skipping; FAP pathogenesis

**1. Introduction**

Familial adenomatous polyposis (FAP; OMIM # 175100) is an autosomal dominant disorder characterized by the development of hundreds to thousands of colorectal adenomatous polyps, which, if left untreated, progress to colorectal cancer (CRC) [1–3]. FAP patients may develop various extracolonic manifestations, including desmoid tumors, gastrointestinal polyposis, hepatoblastoma, thyroid cancer, and other malignancies [4–8]. Based on the number of colorectal polyps, onset age, and extracolonic manifestations, FAP can be classified into four forms: (1) classic FAP with profuse polyposis (>1000 adenomas); (2) classic FAP with intermediate colonic polyposis (100–1000 adenomas); (3) attenuated FAP (AFAP, <100 adenomas); (4) gastric polyposis and desmoid FAP (GD-FAP), which is characterized by less than 50 polyps, a higher risk of developing desmoid tumors, and a greater susceptibility to give rise to diffuse gastric polyposis or adenomas [4,9].

FAP is caused by germline mutations in the tumor suppressor gene APC, which is located on chromosome 5 and comprises 15 translated exons. Depending on the tissues in which it is expressed, APC undergoes alternative splicing leading to multiple protein
variants whose molecular weight varies from 90 to 300 kDa [10–17]. The most abundant 
APC transcript encodes a 2843 amino acid protein [18].

To date, nearly 2000 APC mutations have been described in FAP. The vast majority of 
these mutations are predicted to result in truncated protein products due to nonsense or 
frameshift variants or large genomic deletions [19]. Mutations predicted to result in APC 
aberrant splicing have rarely been reported. Isoforms lacking exon 9 or exon 14 owing to 
splice site mutations have also been associated with FAP disease [20–23].

In this study, we identified and molecularly characterized a novel germline heter- 
zygous splice donor site mutation in APC exon 12 (NM_000038.5: c.1621_1626+7del) 
segregating with the AFAP phenotype in an Italian family. Moreover, we expanded our 
investigation by performing a meta-analysis to correlate all molecularly characterized APC 
exon 12 and exon 13 splicing mutations with FAP clinical phenotypes.

2. Materials and Methods

2.1. Patient Recruitment

The index patient underwent genetic testing following informed consent. Molecular 
testing carried out in this study is based on the routine clinical diagnostic assessment 
performed at our Institute. Written informed consent to perform genetic testing and 
进一步 studies was obtained from the patient using a form approved by the competent 
ethics committee, in line with the principles of the Declaration of Helsinki and any other 
applicable local ethical and legal requirements (protocol code N° 170-date of approval 
31 October 2016).

2.2. Mutation Analysis

Genomic DNA was extracted from peripheral blood with the QIAamp DNA Blood 
Mini Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer’s instructions. APC 
complete coding region was screened for mutations as previously described [24] using 
primer sequences previously published by Groden J et al. [12]. In order to scan the exons 
for mutations, the APC gene was divided into 23 amplicons using specific primer pairs, 
with seven amplicons covering exon 15. Each amplicon was sequenced in forward and 
reverse directions with the same primers used for PCR amplification or internal sequencing 
primers (Table S1). PCR sequencing and capillary electrophoresis were performed on an 
Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). 
Mutations and polymorphisms were confirmed in independently amplified PCR products.
The global population frequency of the identified APC variant was retrieved from the 
1000 Genome [25,26], dbSNP [27,28], gnomAD [29,30], and NHLBI Exome Sequencing 
Project (ESP) [31,32] databases. Moreover, the HGMD Professional [33], InSiGHT [34,35], 
and Clinvar [36,37] databases were interrogated to assess the pathogenicity of the ide- 
ntified variant.

To evaluate the effect of the APC c.1621_1626+7del mutation on RNA splicing, four 
splice site prediction algorithms integrated into Alamut Visual version 2.15 (Sophia Ge- 
netics SAS; Bidart, France) were interrogated simultaneously: Splice Site Finder (SSF), 
MaxEntScan (MES), Splice Site Prediction by Neural Network (NNS), and Gene Splicer 
(GS). The default thresholds of each tool were used for the analysis. A variation of 
more than 10% in at least two algorithms was considered as having an effect on the 
splicing process.

The identified variant was classified according to the American College of Medical 
Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) 
variant classification scheme [38].

2.3. RT-PCR and mRNA Analysis

Total RNA from peripheral blood was extracted with the QIAamp RNA Blood Mini 
Kit (Qiagen) according to the manufacturer’s instructions. One microgram of RNA was 
reverse-transcribed to cDNA using the Maxima H Minus First Strand cDNA Synthe-
sis Kit (Thermo Fisher Scientific). The 5′ and 3′ flanking regions of the APC mutation site (NM_000038.5: c.1621_1626+7del) were amplified using the Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) and the following primers (10 pmol each): APC_Ex10-11_Fw (NM_000038.5) GAATGAACTAGGGGACTACAGGC, and APC_Ex13-14_Rv (NM_000038.5) GGGTTGATTCCTTTTAACTTC. PCR amplification was carried out at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, with a final elongation at 72 °C for 5 min. PCR products were loaded onto 3% agarose gel in 0.5X TBE and visualized using SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). Sequencing and capillary electrophoresis were performed on an Applied Biosystems 3130 Genetic Analyzer.

2.4. Cell Line

The HEK-293 cell line was purchased from ATCC and cultured in DMEM high glucose (HG), without pyruvate (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific), 1% pyruvate (Thermo Fisher Scientific), 1% NEAA (Thermo Fisher Scientific), and 100 U/ml penicillin–streptomycin (Thermo Fisher Scientific) in a 37 °C and 5% CO2 incubator. The cell line was tested to be mycoplasma-free according to Venor®GeM Advance kit (Minerva Biolabs, Berlin, Germany) at multiple times throughout the study.

2.5. Plasmid Construct and Expression

Fragments with the wild type or mutant alleles containing APC exon 12 (NM_000038.5), flanked by upstream (311 nt) and downstream (447 nt) intronic sequences, were amplified using the following primers: Cloning_APC_Fw_EcoRI ACCAGTGAATTCTGACCAAAGGCAAGTTGTACACAC, and Cloning_APC_Rv_BamHI ACCGATGGATCCTCCTAAATGCTACTACGTGCC. Fragments were cloned into the splicing vector pSPL3, linearized with EcoRI and BamHI. All constructs were confirmed by direct sequencing.

2.6. In Vitro Splicing Assay

HEK-293 cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s instruction for 24 h. Cells were harvested, total RNA was extracted with the PureLink™RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions, and used for RT-PCR to confirm splicing patterns. cDNA was synthesized as described above and used as a template for PCR amplification with the following vector-specific primers: SD6_FW GTCTGAGTACCTGGACAACC and SA2_RV GATCTCAGTGGTATTTGTGAGC. PCR amplification was carried out with the Phusion High-Fidelity DNA Polymerase at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 52 °C for 10 s, and 72 °C for 15 s, with a final elongation at 72 °C for 5 min. PCR products were loaded onto 2% agarose gel in 0.5X TBE and visualized using SYBR Safe DNA Gel Stain. Sequencing and capillary electrophoresis were performed on an Applied Biosystems 3130 Genetic Analyzer.

2.7. Meta-Analysis

The meta-analysis of APC splicing mutations was performed on the Human Gene Mutation Database Professional (HGMD Professional; Qiagen), a comprehensive collection of germline mutations in nuclear genes that are associated with human-inherited diseases [33]. We reviewed all the papers identified in the aforementioned database and collected relevant clinical information (i.e., gender, age at diagnosis, gastric or colonic polyposis, and specific APC mutations). Studies including patients without clinical information were excluded.

3. Results

3.1. Clinical History and Genetic Findings

The index case was a 53-year-old male referred to our institution for genetic counseling. The patient presented at 41 years of age with an attenuated colorectal phenotype (AFAP phenotype), which is consistent with the presence of two intestinal polyps. Histological
examination revealed tubular adenomas measuring 4 and 6 mm with moderate-grade
dysplasia. After one year, colonoscopy examination did not show evidence of poly-
posis. The patient was followed up with yearly colonoscopy, which showed three tubular
adenomas (2.5 mm) with moderate-grade dysplasia in the large bowel at the age of 43
and two adenomatous polyps (2 mm) with high-grade dysplasia in the sigmoid colon
at the age of 44. The patient developed small polyps at the age of 48 and numerous sessile
polyps throughout the colon at the age of 51. After one year, colonoscopy examination
revealed the presence of various polypoid and non-polypoid lesions in the transverse and
descending colon. The patient had a positive family history of colorectal cancer and colon
polyposis (Figure 1).
Figure 2. Characterization of APC splicing mutation c.1626_1627+7del. (A) Sequencing electropherograms of genomic DNA from a healthy control individual and the proband, showing the splicing mutation c.1626_1627+7del. (B) Left: Agarose gel electrophoresis showing the RT-PCR analysis of mRNA extracted from peripheral blood of the patient (P) carrying the APC c.1621_1626+7del mutation and a healthy control (C). Amplified products obtained with primers spanning APC exon 10–11 and 13–14 boundaries were separated on 3% agarose gel and independently sequenced. Center: Schematic diagrams showing the wild type amplification product (356 bp) and the altered-splicing amplification product lacking APC exon 12 (277 bp). Right: Sequencing electropherograms of cDNA splicing isoforms generated from the wild type and mutant RT-PCR products. Bottom: Diagram showing the localization of the primers (indicated as arrows) used for RT-PCR experiments.

3.2. Analysis of Patient’s Processed Transcripts

To determine the effect of nucleotide deletion c.1621_1626+7del, total RNA was isolated from peripheral blood of the proband and an unrelated control. The APC transcript between exons 10 and 14 was amplified by RT-PCR, and the obtained fragments were isolated and sequenced (Figure 2B). Gel electrophoresis of the PCR products showed an expected-size fragment (356 bp) both in control and patient samples; however, a lower fragment (277 bp) was also found in the latter. Sequencing analysis of this fragment showed that the c.1621_1626+7del mutation results in the loss of exon 12 splice donor site and exon 12 skipping (Figure 2B). To confirm our results, we performed a minigene study.
using the pSPL3 plasmid. As described in the Section 2, fragments with the wild type or mutant exon 12 (78 bp) allele, flanked by upstream (311 nt) and downstream (447 nt) intronic sequences, were cloned into the splicing vector pSPL3 (Figure 3A). The pSPL3 empty vector, pSPL3_APC_wt, and pSPL3_APC_Δ1621_1626+7 were transfected into HEK-293 cells for 24 hours, and the RNA was collected. Minigene assays showed that the wild type construct resulted in a 341 bp PCR product containing exon 12, while both the empty vector and the mutant construct produced a 263 bp PCR product missing APC exon 12. The obtained fragments were confirmed by sequencing analysis (Figure 3B).

Figure 3. Splicing minigene reporter assay based on the pSPL3 exon-trapping vector. (A) Schematic representation of the pSPL3 minigene reporter used for the molecular characterization of APC splicing mutation c.1621_1626+7del. The pSPL3 vector contains two exons (SD and SA) and a functional intron, with transcription beginning after the SV40 promoter and ending at the late poly(A) signal (LPAS). EcoRI and BamHI indicate the cloning sites used to subclone the genomic APC fragments obtained from the wild type and mutant alleles (c.1621_1626+7del). (B) RT-PCR analysis of transcripts derived from the indicated pSPL3 reporter minigenes transfected in HEK-293 cells. Left: Agarose gel electrophoresis showing the RT-PCR products obtained with the SD6 and SA2 primers from HEK-293 cells transfected with the pSPL3 empty vector (263 bp), the pSPL3 vector with the genomic APC fragment from the wild type allele (341 bp), or the pSPL3 vector with the genomic APC fragment from the mutant allele (263 bp), and untransfected HEK-293 cells (negative control). Center: Schematic diagrams showing the RT-PCR products obtained. Right: Sequencing electropherograms of the RT-PCR products obtained.

3.3. Meta-Analysis

We performed a literature meta-analysis of APC splicing mutations to identify disease-causing splice site mutations that do not change the reading frame of the aberrant transcript.
and to evaluate their effect on transcript processing and patient phenotype. So far, 119 unique APC splicing mutations, including the one characterized in the present study, have been reported in FAP patients. Of these, 69 (58%) have been molecularly characterized at the mRNA level and mainly (60/69, 87%) cause a reading frame shift, while a very small proportion (9/69, 13%) leads to an in-frame APC protein [39–88] (Table S2). Specifically, among the splicing mutations leading to an in-frame protein, 4/9 cause exon 12 or exon 13 skipping with loss of armadillo repeat 2 (ARM2) and armadillo repeat 3 (ARM3) in the APC N-terminal armadillo repeat domain. Of these, 3 were reported in patients with AFAP phenotype [45,54,59] (Table 1). The remaining molecularly characterized splicing mutations leading to an in-frame protein (5/9) result in skipping of exon 5, 7, 8, or (partially) 9 with loss of APC regions not encompassing known functional sites/domains [45,54,67] (Table S3).

Table 1. APC splicing mutations leading to an in-frame protein and skipping of exon 12 or exon 13.

| Gene | Variant (Human Genome Variation Society, HGVS) | Chromosome Position (GRCh37) | Location | Observed Effect on splicing | Effect on mRNA (HGVS) | Effect on Protein (HGVS) | Clinical Phenotype (Classic FAP/AFAP) | Reference |
|------|-----------------------------------------------|-----------------------------|----------|----------------------------|-----------------------|-------------------------|--------------------------------------|----------|
| APC  | c.1621_1626+7del chr5:g.112163698-112163710del | Exon 12/Intron 12           | Exon 12  | Exon 12 skipping r.1549_1626del p.A517_Q542del AFAP Present study |                      |                         | AFAP                                 |          |
| APC  | c.1626G>C chr5:g.112164552G>C                | Exon 12                     | Exon 12  | r.1549_1626del p.A517_Q542del n.d. [45] |                      |                         | AFAP                                 |          |
| APC  | c.1627G>T chr5:g.112164553G>T               | Exon 13                     | Exon 13  | r.1627_1743del p.V543_K581del AFAP [54] |                      |                         | AFAP                                 |          |
| APC  | c.1742A>G chr5:g.112164668A>G               | Exon 13                     | Exon 13  | r.1627_1743del p.V543_K581del AFAP [59] |                      |                         | AFAP                                 |          |

n.d.: not discriminated.

In order to provide further insight into the relationship between APC exon 12 or exon 13 splicing mutations leading to an in-frame protein, the clinical phenotype, and the potential underlying molecular mechanisms in FAP disease, we retrieved clinical and molecular data of FAP patients bearing truncating mutations that lead to partial or total removal of ARM2 and/or ARM3 and disrupt all downstream APC protein domains [50,53,71,89–105] (Table S4). Then, we sought to compare the phenotypic consequences of splicing mutations leading to in-frame amino acid deletions within the ARM2 or ARM3 motifs of the APC protein N-terminal armadillo repeat domain with those of truncating mutations located in the ARM2 (aa 505–547) or ARM3 (aa 548–591) domains leading to partial or total removal of ARM2 and/or ARM3 and disrupting all APC downstream regions (aa 505–2843), including the β-catenin-regulating domains (Figure 4).

A total of 33 patients with data on colon polyposis clinical phenotype and truncating alterations located in the ARM2 and ARM3 domains of APC were identified: (i) 12 patients harbored a truncating mutation involving the ARM2 domain, (ii) four patients harbored a truncating mutation involving the ARM2 and ARM3 domains, and (iii) 17 patients harbored a truncating mutation in the ARM3 domain. In this cohort, the percentage of patients with the classic FAP clinical phenotype was higher (29/33, 87.9%) than the percentage of patients with the attenuated FAP clinical phenotype (4/33, 12.1%). Furthermore, the classic FAP clinical phenotype was only observed in patients with APC truncating mutations, whereas all the patients with splicing mutations leading to in-frame amino acid deletions involving APC ARM2 or ARM3 motifs exhibited the attenuated clinical variant of the disease.
Figure 4. APC coding region. (A) Schematic diagram of wild type APC protein, depicting conserved regions and domains that interact with other proteins. (B) Schematic diagram of APC truncated proteins that result from total removal of ARM2 and/or ARM3 along with disruption of all downstream APC domains and are associated with the classic FAP phenotype. (C) Schematic diagram of APC variants that result from exon 12 or exon 13 splicing mutations leading to an in-frame protein and are associated with the AFAP phenotype.

4. Discussion

RNA splicing is a key cellular process that governs several biological processes, including cellular proliferation, survival, and differentiation [106]. Dysregulation of pre-mRNA splicing is increasingly recognized as an important mechanism that is linked to cancer [107]. In the context of multistep carcinogenesis of CRC, genetic lesions that affect APC splicing are likely to significantly contribute to the etiology of the disease.

APC is a crucial tumor suppressor gene in both sporadic and hereditary CRC. It encodes a large multifunctional protein comprising several motifs and domains, including an oligomerization domain, an ARM domain, a region containing several β-catenin-binding repeats and axin-binding repeats, and a basic domain that interacts with the microtubules. The wild type APC protein plays an important role in Wnt signaling by promoting the degradation of β-catenin. Due to its interaction with a variety of other proteins, APC is also involved in cellular processes related to cell migration, cell adhesion, proliferation, differentiation, and chromosome segregation [108].

APC mutational inactivation is a key event in the development of colon cancer and the intestinal polyp disorder FAP. The severity of the FAP phenotype depends on the location of APC mutations, indicating a complex role for APC that extends beyond the canonical Wnt pathway [9,108].

Most APC disease-causing variants result in a premature termination codon impairing protein function; however, a minor fraction has been found to disrupt the splicing pattern of the gene [4]. In light of the above, the functional characterization and clinical classification of aberrant splicing variants involving the APC gene may support diagnostic accuracy in medical genetics. In this study, we report a novel splicing mutation in the APC tumor suppressor gene. This variant was identified by direct sequencing in an Italian AFAP
family and consists of a small deletion involving the last six nucleotides of exon 12 and seven nucleotides including the splice donor site of intron 12 (c.1621_1626+7del).

The frequency of this mutation was assessed by interrogating various population databases. This analysis revealed that APC c.1621-1626+7del variant is not listed in the dbSNP, 1000 Genome, gnomAD, and ESP databases. To assess the putative effect of this variant on the splicing process, we performed an in silico analysis using splicing prediction tools, which indicated a potential splicing alteration due to the loss of APC exon 12 canonical splice donor site.

To confirm whether this mutation could affect APC splicing, RNA was isolated from the proband, the APC transcript between exon 10 and 14 was amplified by RT-PCR, and the obtained products were isolated and sequenced, revealing the absence of APC exon 12. Moreover, to ascertain the in vivo relevance of the effect of the identified APC mutation on splicing, we performed a minigene splicing assay. Our results showed that APC c.1621-1626+7del variant affects the splicing process, resulting in complete skipping of exon 12. However, this deletion does not disrupt the open reading frame of the aberrant transcript, which lacks some, but not all, Armadillo repeat motifs.

According to ACMG/AMP criteria, our clinical and molecular characterization of the identified variant provides evidence of pathogenicity.

Next, we performed a meta-analysis to investigate the correlation between APC exon 12 or exon 13 splicing mutations that lead to an in-frame protein lacking functional domains/sites and the corresponding clinical phenotypes. To date, nearly 2000 APC mutations have been described in FAP, almost all of which (~87%) lead to loss of function (nonsense mutations, small deletions, small insertions, and gross rearrangements), while only a few (~6%) have been reported to cause or potentially cause impaired splicing of the gene product. The remaining APC mutations are missense mutations and mutations in regulatory regions of the gene (data obtained from HGMD Professional) [33]. Our literature analysis revealed that only a small proportion of these splice site mutations (69/119) have been characterized at the mRNA level, with the vast majority (60/69, 87%) causing a reading frame shift and a tiny fraction (9/69, 13%) leading to an in-frame APC protein with loss of functional domains/sites (Table S2).

Specifically, among the molecularly characterized splicing mutations leading to an in-frame protein, 5/9 cause skipping of exon 5, 7, 8, or (partially) 9 with loss of APC regions not encompassing known functional sites/domains, while 4/9 have been reported to cause the deletion of exon 12 (p.Ala517_Gly542del) or 13 (p.Val543_Lys581) with loss of ARM2 and/or ARM3.

Splicing mutations causing the loss of armadillo functional domains have been reported in patients with FAP disease. Interestingly, deletion of exon 13 (p.Val543_Lys581del) leads to the loss of the last five amino acids of ARM2 and an almost complete loss of ARM3.

Skipping of exon 13 has been reported to be associated with a mutation in a highly conserved splice acceptor site (c.1627G>T, the first base of exon 13) in a patient with AFAP phenotype who underwent subtotal colectomy for carcinoma at the age of 60 [54]. In another report, skipping of exon 13 was found to be caused by a missense mutation in exon 13 (c.1742A>G) that was detected in a patient with attenuated FAP [59]. Splicing mutations resulting in the loss of exon 12 (p.Ala517_Gly542del) lead to an almost complete loss of ARM2. Skipping of exon 12 has been reported to be associated with a mutation in a highly conserved splice donor site (c.1626G>C, the last base of exon 12) in a patient with FAP disease whose clinical phenotype was not described [45].

Based on clinical evaluation (age of manifestation, number and size of polyps, and absence of colorectal cancer until the age of 35), the patient carrying the newly identified APC splicing mutation c.1621_1627+7del described in this study and its family were classified as having an attenuated form of FAP.

Patients with AFAP tend to develop fewer adenomatous polyps, with colorectal tumors occurring at an older age compared with patients with classic FAP. Genotype–
phenotype association studies have revealed that AFAP patients mainly carry mutations at the 5’ end of the gene or at splice junctions involving the alternatively spliced region of exon 9 [4].

Specifically, mutations located in exon 9 alternative splice site have been reported to cause inefficient exon skipping resulting in the generation of a shorter APC isoform along with normal transcripts from the mutant allele [39,43,45,47,54,72]. A recent study investigating the molecular mechanisms leading to AFAP in patients carrying a mutation in the alternatively spliced region of exon 9 has suggested that a “third hit” (somatic mutations of both APC alleles) is necessary for tumorigenesis to occur in these patients [109].

In an effort to elucidate the correlation between mutations causing in-frame loss of functional ARM repeat domains and clinical phenotypes, we compared the clinical and molecular data of FAP patients carrying APC splicing mutations that lead to an in-frame protein lacking ARM2 and/or ARM3 with those bearing APC truncating mutations that result in partial or total removal of ARM2 and/or ARM3 along with disruption of all downstream domains.

Our results demonstrated a trend towards the development of a milder FAP phenotype (attenuated FAP) in patients with splicing mutations in ARM2 and/or ARM3 compared to patients with truncating mutations. The attenuated phenotype observed in patients harboring APC mutations that lead to loss of exon 12 or 13 and cause partial deletion of ARM motifs suggests a potential mechanism of pathogenesis in FAP disease.

ARM domains are abundant in eukaryotic proteins and are characterized by tandem armadillo repeats of approximately 42 amino acids in length that participate in protein-protein interaction. ARM domains are involved in a broad range of important cellular processes, including signal transduction, nuclear transport, and regulation of cytoskeleton formation [110].

The ARM domain located at APC N-terminal is encoded by exons 10–14 and contains seven armadillo repeats that provide a structural platform for interaction with several other proteins, including SAM68 [111], ASEF [112], KAP3 [113], IQGAPI [114], and AMER1 [115]. The structural diversity of these binding partners reveals that APC armadillo repeats may be involved in Wnt signaling, cell–cell adhesion, cell polarization, and cell migration. It has also been reported that the loss of ARM domains results in increased tumor initiation, suggesting a putative tumor-suppressive function for this region [116,117]. Furthermore, previous studies on co-crystal structures showed that the replacement of APC key residues, such as N507K, N550K, N594K, and K516/E, abolishes the association between APC-ARM and AMER1-A1/A2/A4 [115], ASEF [118], and SAM68 [111]. These APC ligands show no apparent sequence similarity, nor do they have any resemblance with other APC-binding motifs. However, in their physical interaction with APC, these proteins occupy the same surface groove within APC-ARM domains and assume the same antiparallel position with respect to armadillo repeats [115].

The evidence that addition of the ASEF protein to preassembled APC-ARM/A MER1 complexes progressively dissociates APC-ARM from AMER1 in a dose-dependent manner confirms the hypothesis that these APC partners can compete with each other in a mutually exclusive manner [115].

Consistently, from a functional point of view, AMER1 and SAM68 seem to have antithetical roles in the regulation of the Wnt pathway. In particular, AMER1 negatively regulates Wnt signal transduction by promoting ubiquitination and degradation of β-catenin [119], while recent data showed that aberrant upregulation of SAM68 induces cancer cell proliferation in vitro by activating the Wnt/β-catenin signaling pathway [120].

Note that it has also been reported that the complex between APC and SAM68 regulates the alternative splicing of members of the T cell factor (TCF) family of transcription factors that associate with β-catenin, in the presence of the Wnt signal or in the absence of APC, in order to regulate the expression of Wnt target genes involved in tumor formation. APC mutations that truncate regions downstream of the ARM domain lead to the accumulation of the TCF-1E splice variant, which strongly transactivates Wnt target genes [111].
Note also that genetic disease mutations can have an impact on protein conformational equilibria and dynamics [121,122]. Recently, the missense APC N1026S variant identified in an AFAP family has been predicted to change the conformational flexibility of APC protein, preventing it from establishing stable contacts with β-catenin protein [123].

In this light, another possible explanation as to why mutations causing partial deletion of APC ARM1 and/or ARM2 motifs could produce an attenuated phenotype is that these deletions may induce conformational changes in protein structure and dynamics resulting in impaired physical interaction between APC and its binding partners, including β-catenin. It is therefore tempting to speculate that APC exon 12 or exon 13 splicing mutations leading to the deletion of ARM motifs may decrease APC binding to β-catenin, thereby preventing, at least in part, the export of nuclear β-catenin. This would in turn result in an attenuated clinical variant of FAP disease. Conversely, truncating mutations located in the ARM2 and/or ARM3 motifs lead to complete lack of regulation of the β-catenin protein, causing the classic clinical variant of FAP disease.

For these reasons, APC exon 12 or exon 13 splicing mutations leading to the partial loss of ARM motifs are expected to account for the observed AFAP phenotype in the patients considered in the present study.

5. Conclusions

Altogether, the presented evidence supports mechanism for FAP pathogenesis involving mutations that affect APC ARM domains but do not cause the loss of the seven β-catenin-downregulating 20 amino acid repeats distributed in the central region of the protein. The underlying mechanism of pathogenesis most likely involves binding partners and functions of APC ARM motifs, suggesting that these domains mediate APC tumor suppressor activity and may play a role in carcinogenesis in FAP patients. In conclusion, our findings support a pathogenic role for APC exon 12 or exon 13 splicing mutations in the AFAP phenotype.

**Supplementary Materials:** The following supplementary figure and tables are available online at https://www.mdpi.com/2073-4425/12/3/353/s1. Figure S1: Screenshot from the Alamut software. Splicing effect window around APC c.1621_1626+7del mutation. The top box represents APC wild-type sequence; the bottom box represents APC mutated sequence with the c.1621_1626+7del deletion. The dark blue bars represent the predicted splice donor site. The diagram reveals the abolition of the canonical splice donor site at position c.1626. All four tools predicted that the identified variant abolishes the canonical splice donor site. Table S1: Primers used for genomic PCR amplification and sequencing reactions; Table S2: APC splicing mutations; Table S3: APC splicing mutations leading to an in-frame protein and loss of APC regions not encompassing known functional sites/domains; Table S4: Truncating mutations located in the ARM2 or ARM3 domain of the APC protein.

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