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Article

APC splicing mutations leading to in-frame exon 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 123 unique *APC* splice site 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 4 mutations causing skipping of exon 12 and/or 13 with loss of armadillo repeat 2 (ARM2) and 3 (ARM3), and 5 mutations leading to skipping of exon 5, 7, 8, and (partially) 9 with loss of regions not encompassing known functional domains. The *APC* splicing mutations considered in this study cluster with the AFAP phenotype and delineate a novel molecular mechanism of pathogenesis in FAP disease.

Keywords: Familial Adenomatous Polyposis; APC; Splicing; Exon Skipping; FAP Pathogenesis

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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 profuse 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 16 translated exons. In normal tissues, *APC* undergoes alternative splicing involving non-coding exons 0.1, 0.2, 0.3 [10, 11] and coding exons 1, 3-4, 7, 9, 10A, and 14 [11, 12-17], leading to multiple protein variants whose molecular weight varies from 90 to 300 kDa. The most abundant *APC* transcript lacks the smallest exon, 10A, and 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 heterozygous splice acceptor 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 known *APC* splice site 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 further 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 (approval code N° 170/31/10/2016).

2.2 Mutation analysis

Genomic DNA was extracted from peripheral blood with the QIAamp DNA Blood Mini Kit (Qiagen, Carlsbad, California, USA) according to the manufacturer's instructions. *APC* complete coding region was screened for mutations as previously described [24]. Sequencing and capillary electrophoresis were performed on an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, 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], dbSNP [26], gnomAD [27], and NHLBI Exome Sequencing Project (ESP) [28] databases. Moreover, the HGMD Professional [29], InSiGHT [30], and Clinvar [31] databases were interrogated to assess the pathogenicity of the identified variant.

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2.3 RT-PCR and mRNA analysis

Total RNA from peripheral blood was extracted with the QIAamp RNA Blood Mini Kit (Qiagen, Carlsbad, California, USA) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed to cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The 5′ and 3′ flanking regions of the *APC* mutation site (NM_000038.6**:** c.1621_1626+7del) were amplified using the Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the following primers (10 pmol each): APC_Ex10-11_Fw (NM_000038.6) GAATGAACTAGGGGGACTACAGGC and APC_Ex13-14_Rv (NM_000038.6) GGGTTGATTCCTTTTTAACTTC. 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, Waltham, Massachusetts, USA). 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, Waltham, Massachusetts, USA) with 10% FBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1% pyruvate (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1% NEAA (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) 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 ACCAGTGAATTCGACCAAGGCAAGTGTTACACAC and Cloning_APC_Rv_BamHI ACCGATGGATCCTCCTAAATGCTACTACAGTGCC. 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, Waltham, Massachusetts, USA) 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, Waltham, Massachusetts, USA) according to the manufacturer's instruction, 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 GTCTGAGTCACCTGGACAACC 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.

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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 [32]. In order to better define the phenotypes associated with germline splicing mutations resulting in exon skipping and partial loss of functional APC protein domains, we did not include in our subsequent analysis splicing mutations leading to a truncated null APC protein. 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, and had a positive family history of colorectal cancer and colon polyposis (Figure 1).

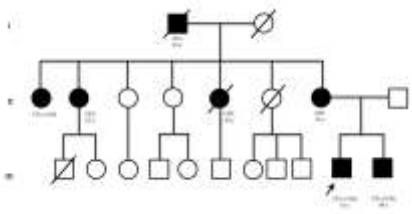

Figure 1. Pedigree of the Italian family involved in this study. Squares indicate men, circles represent women. The arrow indicates the index case. Unfilled symbols indicate unaffected individuals. Slashed symbols denote dead individuals. Filled symbols denote individuals with AFAP. The following information is given below each filled symbol: clinical manifestations (CRC = colorectal cancer; CPs = colon polyps (the number of polyps is indicated); Pca = prostate cancer, age at diagnosis (y = years).

All analyzed polyps were initially classified as tubular with moderate-grade dysplasia. The patient underwent regular follow-up with annual colonoscopies, which showed the presence of intestinal polypoid lesions. Histological examination of surgically resected polyps revealed tubular adenomas with moderate or high-grade dysplasia. The brother of the proband exhibited signs of attenuated FAP, and his mother developed colorectal carcinoma at the age of 65. The *APC* mutation analysis performed on the proband's DNA revealed a heterozygous splicing mutation in *APC* exon 12 (NM_000038.5: c.1621_1626+7del) (Figure 2a).

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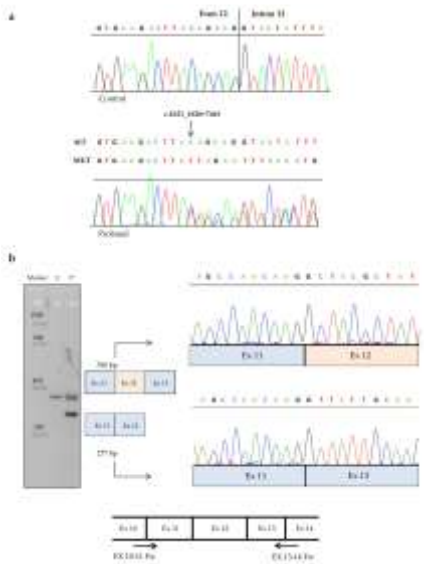

Figure 2. Characterization of *APC* splicing mutation c.1626_1627+7del. (**a**) Sequencing electropherograms of genomic DNA from a healthy control individual and the proband, showing the splicing mutation c.1626_1627+7del. (**b**) Left: Agarose gel electrophoresis showing the RT-PCR analysis of mRNA 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.

The identified variant was found to be rare since it was not listed in global population databases (1000 Genome, Exome Aggregation Consortium, NHLBI Exome Sequencing Project). Moreover, this variant has never been reported in major disease-associated databases (HGMD, LOVD, InSiGHT, and Clinvar).

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 Materials and Methods section, 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). 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).

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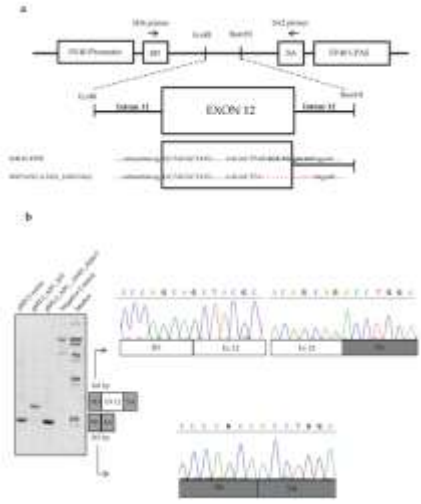

Figure 3: Splicing minigene reporter assay based on the pSPL3 exon-trapping vector. **(a)** Schematic representation of the pSPL3 minigene reporter used for the molecular characterization of *APC* splicing mutation c.1621_1626+7del. The pSPL3 vector contains two exons (SD and SA) and a functional intron, with transcription beginning after the SV40 promoter and ending at the late poly(A) signal (LPAS). EcoRI and BamHI indicate 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, 123 unique *APC* splice site mutations, including the one characterized in the present study, have been reported in FAP patients. Of these, 69 (56%) 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 (Supplementary Table 1). Specifically, among the splicing mutations leading to an in-frame protein, 4/9 were reported in patients with AFAP phenotype and cause exon 12 and/or exon 13 skipping with loss of armadillo repeat 2 (ARM2) and armadillo repeat 3 (ARM3) in the APC N-terminal armadillo repeat domain. The remaining molecularly-characterized splicing mutations leading to an in-frame protein (5/9) result in skipping of exon 5, 7, 8, and (partially) 9 with loss of APC regions not encompassing known functional sites/domains (Table 1).

Table 1. *APC* splicing mutations leading to an in-frame protein.

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n.a.: not available

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	Reference
APC	c.645+1G>T	chr5:g.112116601G>T	Intron 5	Exon 5 skipping	r.532_645del	p.F178_Q215del	n.a.	[54]
APC	c.730-3C>G	chr5:g.112136973C>G	Intron 6	Exon 7 skipping	r.730_834del	p.R244_Q278del	n.a.	[38]
APC	c.933G>C	chr5:g.112151290C>G	Exon 8	Exon 8 skipping	r.835_933del	p.G279_K311del	n.a.	[61]
APC	c.1240C>T	chr5:g.112154969C>T	Exon 9	Exon 9 partial skipping	r.934_1236del	p.V312_Q412del	n.a.	[61]
APC	c.1242C>T	chr5:g.112154971C>T	Exon 9	Exon 9 partial skipping	r.934_1236del	p.V312_Q412del	n.a.	[61]
APC	c.1621_1626+7del	chr5:g.112163698-112163710del	Exon 12/ Intron 12	Exon 12 skipping	r.1549_1626del	p.A517_Q542del	AFAP	Present study
APC	c.1626G>C	chr5:g.112164552G>C	Exon 12	Exon 12 skipping	r.1549_1626del	p.A517_Q542del	n.a.	[38]
APC	c.1627G>T	chr5:g.112164553G>T	Exon 13	Exon 13 skipping	r.1627_1743del	p.V543_K581del	AFAP	[54]
APC	c.1742A>G	chr5:g.112164668A>G	Exon 13	Exon 13 skipping	r.1627_1743del	p.V543_K581del	AFAP	[48]

In order to provide further insight into the relationship between *APC* 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 (Supplementary Table 2). Then, we sought to compare the phenotypic consequences of splicing mutations leading to in-frame amino acid deletions within the ARM2 (aa 517–542) or ARM3 (aa 543–581) motifs of the APC protein N-terminal armadillo repeat domain with those of truncating mutations located in the ARM2 or ARM3 domains leading to partial or total removal of ARM2 and/or ARM3 and disrupting all APC downstream regions, including the β-catenin-regulating domains (aa 517–2843).

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) 4 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 (27/33, 81,8%) compared to patients with the attenuated FAP clinical phenotype (6/33, 18,2%). Furthermore, the classic FAP clinical phenotype was only observed in patients with *APC* truncating mutations, whereas all the patients with splicing mutations leading to an in-frame APC protein exhibited the attenuated clinical variant of the disease.

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4. Discussion

RNA splicing is a key cellular process that governs several biological processes, including cellular proliferation, survival, and differentiation [101]. Dysregulation of pre-mRNA splicing is increasingly recognized as an important mechanism that is linked to cancer [102]. 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. 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 close to 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 the *APC* c.1621-1626+7del variant is not listed in the dbSNP, 1000 Genome, gnomAD, and ESP databases, suggesting that it is most likely pathogenic.

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, a minigene splicing assay showed that the c.1621-1626+7del *APC* 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.

Next, we performed a meta-analysis to investigate the correlation between *APC* 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. Our literature analysis revealed that only a small proportion of these splice site mutations (69/123) 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 (Supplementary Table 1).

Specifically, among the molecularly characterized splicing mutations leading to an in-frame protein, 5/9 cause skipping of exon 5, 7, 8, and (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 5 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 [48]. 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 [38].

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Based on clinical evaluation (age of manifestation, number and size of polyps, absence of colorectal cancer until the age of 35), the members of the family described in this study carrying the newly identified *APC* splicing mutation c.1621_1627+7del 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 [33, 38, 40, 45, 54, 64]. 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 [103].

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 novel potential mechanism of pathogenesis in FAP disease.

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 [104], ASEF [105], KAP3 [106], IQGAP1[107], and AMER1 [108]. 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 [109-110]. 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 [108], ASEF [111], and SAM68 [104]. 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 [108].

The evidence that addition of the Asef protein to pre-assembled APC–ARM/Amer1 complexes progressively dissociates APC–ARM fromAMER1 in a dose-dependent manner confirms the hypothesis that these APC partners can compete with each other in a mutually exclusive manner [108].

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 [112], while recent data showed that aberrant upregulation of SAM68 induces cancer cell proliferation *in vitro* by activating the Wnt/β-catenin signaling pathway [113]. Noteworthy, 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 [104].

Altogether, the presented evidence supports a novel mechanism for FAP pathogenesis, which involves mutations that affect APC ARM domains but do not impact the activity 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.

Supplementary Materials: The following tables are available online at www.mdpi.com/xxx/s1, Table S1: *APC* splicing mutations, Table S2: Truncating mutations located in the ARM2 or ARM3 domain of the APC protein.

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