Synonymous mutation adenomatous polyposis coli$^{\Delta 486s}$ affects exon splicing and may predispose patients to adenomatous polyposis coli/mutY DNA glycosylase mutation-negative familial adenomatous polyposis

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Abstract. Familial adenomatous polyposis (FAP) is an autosomal dominant-inherited colorectal cancer. Recent advances in genetics have indicated that the majority of patients with FAP carry germline mutations of the adenomatous polyposis coli (APC) and mutY DNA glycosylase (MUTYH) genes. However, a large subset of families with a history of FAP have undetectable pathogenic alterations, termed APC/MUTYH mutation-negative FAP. To investigate the germline mutations in the APC and MUTYH genes in Chinese patients with FAP, 13 unrelated patients were enrolled. Through genetic sequencing, four known pathogenic alterations (Lys1061LysfsTer2, Glu1309AspfsTer4, Arg283Ter and Ser1196Ter) of APC and two novel disease-associated pathogenic mutations (Tyr152Ter and Ter522Gly) in MUTYH were identified in six individuals. For samples that did not present with pathogenic alterations, the functional effects of missense, synonymous and intronic mutations were analyzed using bioinformatics tools and databases. Bioinformatics prediction suggested that the synonymous mutation Tyr486Tyr in APC (APC$^{\Delta 486s}$) was likely a disease-causing polymorphism and may have induced the exon skipping of APC. A hybrid mini-gene assay was performed, which confirmed that the synonymous single nucleotide polymorphism APC$^{\Delta 486s}$ induced major splicing defects with skipping of exon 12 in APC. The data of the present study suggested that the synonymous polymorphism APC$^{\Delta 486s}$ was a potential pathogenic alteration that predisposed APC/MUTYH mutation-negative patients to FAP.

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Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant-inherited colorectal cancer syndrome with poor prognosis (1,2). It is well established that FAP is linked to germline mutations of the adenomatous polyposis coli (APC; transcript ID, ENST00000257430.8; https://www.ensembl.org/Homo_sapiens/Transcript/Summary?g=ENSG00000134982; r=5:112737888-112846239;t=ENST00000257430) gene (3,4), and screening for APC germline mutations in families with FAP is a standard and effective tool for the predictive testing of at-risk individuals (3-6). However, through conventional screening methods, a number of patients with FAP have no detectable APC mutations, which is defined as APC mutation-negative FAP (7,8). Certain patients with FAP without detectable germline APC mutations were revealed to carry biallelic mutations in the base-excision-repair gene mutY DNA glycosylase (MUTYH; transcript ID, ENST00000354383.10), and investigators demonstrated that mutations in MUTYH may explain up to 25% of APC mutation-negative FAP cases (8,9). However, a large subset of families with a history of FAP have pathogenic alterations that are undetectable, and this is termed APC/MUTYH mutation-negative FAP (8-10).

In the present study, in order to investigate the germline mutations and the potential causes of APC/MUTYH mutation-negative FAP, a cohort of 13 patients with FAP were enrolled. First, whole-gene sequencing of APC and MUTYH was applied to determine the pathogenic alterations predisposing patients to FAP (11-13), and the results identified four known pathogenic mutations in APC and two novel disease-associated pathogenic mutations in six individuals. For the APC/MUTYH mutation-negative FAP cases, missense, synonymous and intronic mutations in APC and MUTYH

were analyzed using bioinformatics assessment tools and databases; notably, a synonymous mutation of Tyr486Tyr in APC ($APC^{\Delta 486s}$) was predicted to be a disease-causing polymorphism. In-depth analysis of the results suggested that this mutation may have caused exon skipping in APC and that the truncated APC protein resulted in FAP.

It has been established that the majority of synonymous and intronic mutations are silent mutations; however, previous investigations have also revealed that certain synonymous substitutions are associated with a risk of disease and other complex traits including susceptibility to cancer (14,15). Increasing amounts of evidence indicate that synonymous mutations may induce major splicing defects at the mRNA level and consequently result in disease (16).

Combined with the above conclusions and the findings of the present study, it was hypothesized that $APC^{\Delta 486s}$ which was previously considered to be non-pathogenic polymorphism was a potential pathogenic mutation for APC/MUTYH mutation-negative FAP. To confirm the splicing regulation of the synonymous mutation $APC^{\Delta 486s}$ *in vitro*, an exon skipping assay was performed to verify whether the synonymous mutation $APC^{\Delta 486s}$ leads to APC protein truncation. It is hoped that following further studies on the mechanism of exon skipping of the synonymous mutation APCΔ486s, it could be applied as a potential pathogenic alteration in the diagnostic application for those APC/MUTYH mutation-negative patients to FAP.

Materials and methods

Ethics statement. The present study was approved by and performed in accordance with the Research Ethics Board of Kunming Medical University (Kunming, China). All participants provided written informed consent, and the ethics committees approved the consent procedure.

Patients. Between January 2013 and December 2017, 13 patients [male:female 8:5, mean age 31 years (range, 16-41] clinically diagnosed with FAP were recruited from the First Affiliated Hospital of Kunming Medical University (Kunming, China). The diagnosis of all patients referred for the present study was confirmed by surgery, and all patients had a family history suggestive of FAP. Blood samples were collected from all patients prior to surgery.

Gene-sequencing of APC and MUTYH. Blood samples from all patients with FAP underwent exon-specific DNA sequencing for APC and DNA was purified following the manufacturer's protocols of the DNA extraction kit (QIAamp DNA blood mini kit; Qiagen, Valencia, CA, USA). The extracted DNA was quantified by a Pearl nanophotometer (Implen, Munich, Germany). The promoter region, exons 1-14 and 21 segments of exon 15 in the APC gene were separately amplified by polymerase chain reaction (PCR) (11,13,17). Following the DNA sequencing of APC, whole-gene sequencing of MUTYH was performed for the APC mutation-negative FAP samples. The primer pairs used for MUTYH sequencing in the present study were designed as previously described (12,13).

In silico analysis and functional single nucleotide polymorphism (SNP) assessment. The sequencing data for each gene was initially analyzed using Mutation Surveyor (18) (www.softgenetics.com). Human Gene Mutation Database (19) (www.hgmd.org), International HapMap Project (20) (ftp.ncbi.nlm.nih.gov/hapmap/), dbSNP database (21) (www.ncbi.nlm.nih.gov/SNP), 1000 Genomes (http://www.internationalgenome.org/), and Ensembl (22) (www.ensembl.org) were also used.

For APC gene mutations, the Leiden Open Variation Database (LOVD; databases.lovd.nl/shared/genes/APC), BIPMed SNP Array (bipmed.iqm.unicamp.br/snparray/genes/APC), ClinVar at the National Center for Biotechnology Information [NCBI; www.ncbi.nlm.nih.gov/clinvar/?term=APC(gene)], the Universal Mutation Database (UMD)-APC mutations database (www.umd.be/APC/W_APC), Iran Variation Database (genet.ir/variome/genes/APC), International Society for Gastrointestinal Hereditary Tumours Database (www.insight-database.org/genes/APC), LOVD (proteomics.bio21.unimelb.edu.au/lovd/genes/APC), Spain Mutation Database (lovd3.isciii.es/genes/APC) and the Zhejiang University Center for Genetic and Genomic Medicine (23) (www.genomed.org/lovd2/home.php?select_db=APC) were applied.

For the mutation screening of the MUTYH gene, the LOVD (proteomics.bio21.unimelb.edu.au/lovd/genes/MUTYH), Shared database (databases.lovd.nl/shared/genes/MUTYH), UMD-MUTYH mutations database (www.umd.be/MUTYH/), Iran Variation Database (genet.ir/variome/genes/MUTYH), ClinVar at NCBI [www.ncbi.nlm.nih.gov/clinvar/?term=MUTYH(gene)], International Society for Gastrointestinal Hereditary Tumours Database (www.insight-database.org/genes/MUTYH) and PharmGKB (https://www.pharmgkb.org/gene/PA31328/variantAnnotation) were used.

Bioinformatics predictions of the missense, synonymous and intronic substitutions in APC/MUTYH mutation-negative FAP. To investigate the possible genetic causes in patients with APC/MUTYH mutation-negative FAP, bioinformatics prediction tools were used to study the function of any identified missense, synonymous and intron substitutions. Polymorphism phenotype version 2 (24), Sorting Intolerant From Tolerant (SIFT) (25) and SNPs&Gene Ontology (GO) (26) were used to predict deleterious missense substitutions. Exonic splicing enhancer (ESE) finder 3.0 (27) (krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), RESCUE-ESE (28) (genes.mit.edu/burgelab/rescue-ese), exonic-splicing regulatory (ESR) search (26) and putative exonic splicing enhancers (PESX) (29) were used to determine the corresponding functional impacts of the synonymous and intronic substitutions.

Hybrid mini-gene assay of synonymous SNP $APC^{\Delta 486s}$

Generation of mini-gene constructs of synonymous SNP $APC^{\Delta 486s}$. Through bioinformatics analysis, the synonymous and intronic variants in MUTYH were predicted to have no effect on the disease. However, five synonymous SNPs in APC were predicted to be disease-associated mutations (Fig. 1), and among them, $APC^{\Delta 486s}$ was predicted to be associated with exon skipping in APC. To confirm the splicing regulation of the synonymous mutation $APC^{\Delta 486s}$ *in vitro*, a mini-gene system of $APC^{\Delta 486s}$ was constructed to investigate its splicing impact on the APC gene. The nucleotide sequence of exon 12

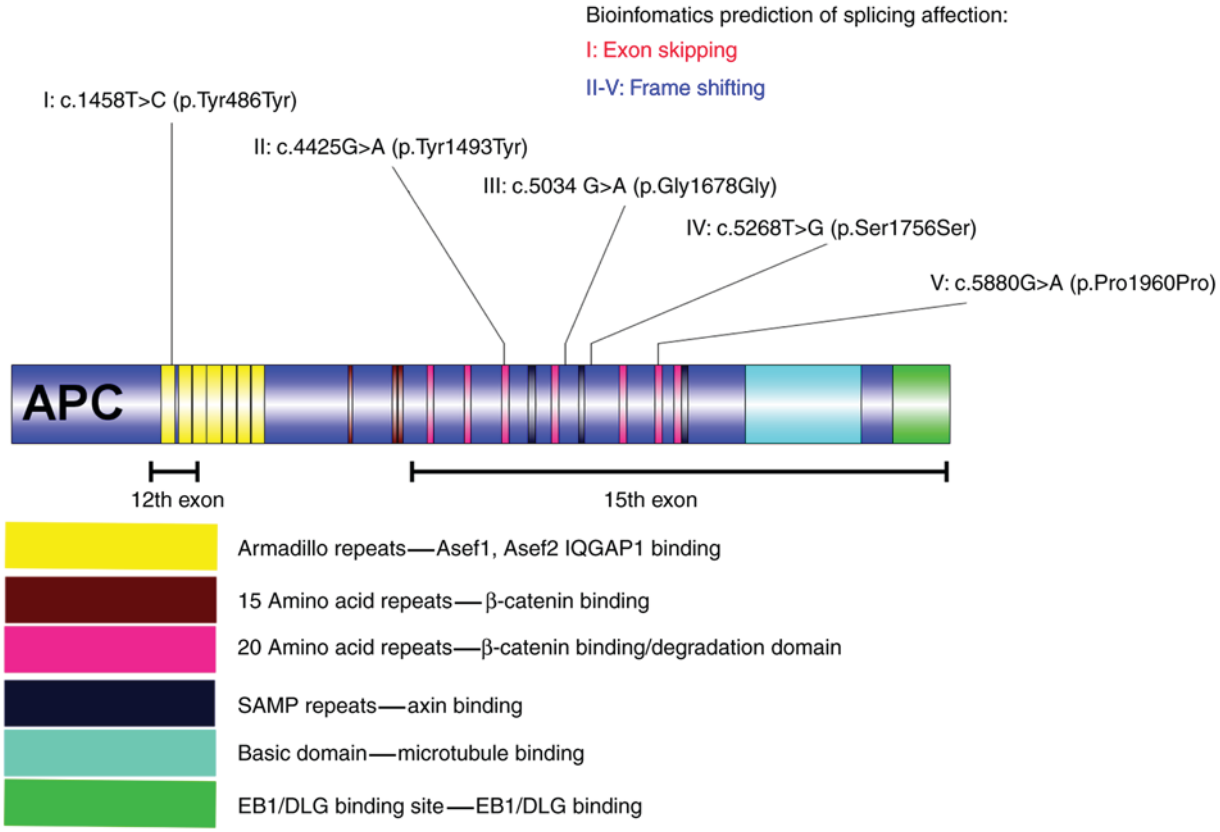

Figure 1. Synonymous substitutions in the APC gene and the corresponding bioinformatics prediction of each mutant polymorphism. I indicates the synonymous substitution c.1458T>C, located in the first armadillo repeat of APC, which is in the Asef1, Asef2 and IQGAP binding domain. The splicing impact of c.1458T>C may have caused the skipping of exon 12 in APC. II indicates the synonymous substitution c.4425G>A located in the third 20 amino acid repeat of APC, which is located in the β-catenin binding/degradation domain. The splicing impact of c.4425G>A may have caused a frameshift mutation in APC. III indicates the synonymous substitution c.5034 G>A is located behind the fourth 20 amino acid repeat of APC, which is in the β-catenin binding/degradation domain. The splicing impact of c.5034 G>A may have resulted in a frameshift mutation. IV indicates the synonymous substitution c.5268T>G located behind the second SAMP repeat of APC. The splicing impact of c.5268T>G may also result in a frameshift mutation. V indicates the synonymous substitution c.5880G>A, located in the sixth 20 amino acid repeat of APC, which is in the β-catenin binding/degradation domain. The splicing impact of c. 5880G>A may have caused a frameshift mutation in APC. APC, adenomatous polyposis coli; Asef, APC-stimulated guanine nucleotide exchange factor; IQGAP1, IQ motif containing GTPase activating protein 1; EB1, microtubule associated protein RP/EB family member 1; DLG, discs large MAGUK scaffold protein 1.

with wild-type and mutant APC$^{\Delta 486s}$ was synthesized, together with 264 bp of the 12th flanking intron, carrying the *Xho*I restriction site, and 210 bp of the 13th flanking intron, carrying the *Hind*III restriction site (Fig. 2), as described previously (30). The wild-type and mutant mini-gene were inserted into pEGFP-N1 to construct the recombinant plasmids pEGFP-N1-wt-mini-gene and pEGFP-N1-mt-mini-gene, respectively (Takara Bio USA, Inc., CA, USA).

Transfection and reverse transcription (RT)-PCR. Following identification by plasmid sequencing (30), the wild-type and mutant mini-gene constructs were transiently transfected into HeLa cells using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to manufacturer's protocol: 1x10^5 HeLa cells were seeded in each 12-well plate and then cultured in DMEM (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 100 ml/l fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.) and 50 ml/l CO_2 at 37˚C. 24 h following transfection, the cells were collected. Total RNA was extracted using the TriPure Isolation reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and analyzed by RT-PCR (2XTSINGKE Master Mix, TSINGKE Biological Technology, Beijing, China). The

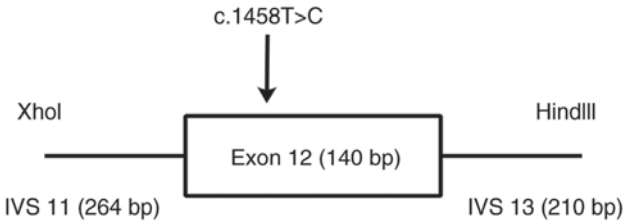

Figure 2. Mini-gene system encoding wild-type and mutant c.1458T>C. The nucleotide sequence of the exon 12 with wild-type and mutant APC$^{\Delta 486s}$, together with 264 bp of flanking intron 12, carrying the *Xho*I restriction site, and 210 bp of flanking intron 13, carrying the *Hind*III restriction site. APC, adenomatous polyposis coli; bp, base pairs; IVS, intervening sequence.

resulting cDNA was amplified by PCR (30 cycles) using the following primers: APC forward, 5'-ATTATTTCGCTCAGC AAGATAAG-3' and reverse, 5'-TTCCATCTGTAGATG TACCTTTGC-3'. GAPDH was selected as reference and the corresponding premiers were: GAPDH forward, 5'-TGAAGG TCGGAGTCAACGG-3' and reverse, 5'-TCCTGGAAGATG GTGATGGG-3'. The PCR thermocycling conditions included an initial denaturation step at 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 sec, 59˚C for 30 sec and 72˚C for 30 sec,

Table I. Variants identified in the patient cohort by Mutation Surveyor.

A, APC

Variant	Functional class
c.3183_3187delACAAA (p.Lys1061LysfsTer2)	Frameshift mutations
c.3927_3931delAAAGA (p.Glu1309AspfsX4)	Frameshift mutations
c.847C>T (p.Arg283Ter)	Nonsense mutation
c.3587C>A (p.Ser1196Ter)	Nonsense mutation
c.2350T>A (p.Ser784Thr)	Missense mutation
c.2638A>C (p.Ile880Leu)	Missense mutation
c.3511C>G (p.Arg1171Gly)	Missense mutation
c.3775A>C (p.Ile1259Leu)	Missense mutation
c.4557T>A (p.Asp1519Glu)	Missense mutation
c.4655A>T (p.Glu1552Val)	Missense mutation
c.6646C>A (p.Pro2216Thr)	Missense mutation
c.220+35T>A	Intronic mutation
c.645+129A>C	Intronic mutation
c.1548+133C>A	Intronic mutation
c.1458T>C (p.Tyr486Tyr)	Synonymous mutation
c.4425G>A (p.Tyr1493Tyr)	Synonymous mutation
c.5034 G>A (p.Gly1678Gly)	Synonymous mutation
c.5268T>G (p.Ser1756Ser)	Synonymous mutation
c. 5880G>A (p.Pro1960Pro)	Synonymous mutation

B, MUTYH

Variant	Functional class
c.456T>A (p.Tyr152Ter)	Nonsense mutation
c.1564T>G (p.Ter522Gly)	Nonstop mutation
c.553G>C (p.Glu185Gln)	Missense mutation
c.1232A>T (p.Gln411Leu)	Missense mutation
c.1268G>A (p.Arg423His)	Missense mutation
c.1483C>G (p.Gln495Glu)	Missense mutation
c.264+11G>A	Intronic mutation
c.304+23G>A	Intronic mutation
c.420+35A>G	Intronic mutation
c.1566+33_1566+35het_delTGT	Intronic mutation
c.423G>A (p.Glu141Glu)	Synonymous mutation
c.450C>A (p.Glu150Glu)	Synonymous mutation
c.1347G>C (p.Tyr449Tyr)	Synonymous mutation

APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis.

and a final 5 min extension step at 72˚C. PCR products were separated by electrophoresis on 2.5% agarose gels containing ethidium bromide, and visualized by exposure to ultraviolet light. The experiments were repeated 3 times.

Results

Micromutations of APC and MUTYH. Following DNA sequencing of APC and MUTYH, micromutations were analyzed using Mutation Surveyor (Table I). For APC, four disease-associated pathogenic alterations (Lys1061LysfsTer2, Glu1309AspfsTer4, Arg283Ter and Ser1196Ter) were identified, which had been previously reported to be pathogenic mutations (31-33,13). In addition to the pathogenic mutations mentioned above, seven missense mutations (Ser784Thr, Ile880Leu, Arg1171Gly, Ile1259Leu, Asp1519Glu, Glu1552Val and Pro2216Thr), five synonymous substitutions (Tyr486Tyr, Tyr1493Tyr, Gly1678Gly, Ser1756Ser and Pro1960Pro) and

Table II. Computational predictions of deleterious missense mutations.

A, APC

Variant	Functional class	PolyPhen-2	SIFT	SNPs&GO
c.2350T>A (p.Ser784Thr)	Missense mutation	BENIGN (0.017)	Tolerated (0.41)	Neutral (RI: 5)
c.2638A>C (p.Ile880Leu)	Missense mutation	BENIGN (0.376)	Tolerated (0.92)	Disease (RI: 6)
c.3511C>G (p.Arg1171Gly)	Missense mutation	BENIGN (0.000)	Tolerated (0.11)	Disease (RI: 1)
c.3775A>C (p.Ile1259Leu)	Missense mutation	BENIGN (0.008)	Tolerated (0.58)	Disease (RI: 7)
c.4557T>A (p.Asp1519Glu)	Missense mutation	N/A	Tolerated (0.16)	Disease (RI: 6)
c.4655A>T (p.Glu1552Val)	Missense mutation	BENIGN (0.104)	Tolerated (0.24)	Neutral (RI: 1)
c.6646C>A (p.Pro2216Thr)	Missense mutation	BENIGN (0.000)	Tolerated (0.12)	Neutral (RI: 0)

B, MUTYH

Variant	Functional class	PolyPhen-2	SIFT	SNPs&GO
c.553G>C (p.Glu185Gln)	Missense mutation	BENIGN (0.020)	Tolerated (0.08)	Neutral (RI: 8)
c.1232A>T (p.Gln411Leu)	Missense mutation	BENIGN (0.000)	Tolerated (0.10)	Neutral (RI: 9)
c.1268G>A (p.Arg423His)	Missense mutation	BENIGN (0.001)	Tolerated (0.12)	Neutral (RI: 4)
c.1483C>G (p.Gln495Glu)	Missense mutation	BENIGN (0.146)	Tolerated (1.00)	Neutral (RI: 9)

PolyPhen-2, >0.50 is predicted to be damaging; SIFT, <0.05 is predicted to be deleterious; RI, prediction reliability scored between 0 (unreliable) and 10 (reliable). Disease, disease-associated polymorphism; neutral, neutral polymorphism; PolyPhen, polymorphism phenotype; SIFT, Sorting Intolerant From Tolerant; SNPs&GO, Single Nucleotide Polymorphisms & Gene Ontology; RI, reliability index; APC, adenomatous polyposis coli; MUTYH, mutY DNA glycosylase.

three intronic mutations (c.220+35T>A, c.645+129A>C and c.1548+133C>A) were identified.

For MUTYH, one novel nonsense mutation (Tyr152Ter) and one nonstop mutation (Ter522Gly) were identified as pathogenic mutations and were predicted to cause premature protein truncation and the continued translation of an mRNA, respectively. In addition, four missense mutations (Glu185Gln, Gln411Leu, Arg423His and Gln495Glu), three synonymous substitutions (Glu141Glu, Glu150Glu and Tyr449Tyr) and four intronic mutations (c.264+11G>A, c.304+23G>A, c.420+35A>G and c.1566+33_1566+35het_delTGT) were identified.

Computational prediction of missense substitutions and 'silent' mutations. Computational prediction suggested that no missense substitutions in APC or MUTYH in the cohort of the present study were disease-associated variants (Table II). The synonymous and intronic substitutions of APC and MUTYH were further analyzed, and the *in-silico* analysis results indicated that the synonymous substitutions (Tyr486Tyr, Tyr1493Tyr, Gly1678Gly, Ser1756Ser and Pro1960Pro) of APC may have affected splicing regulation by creating or removing ESEs or exon splicing silencers. Through bioinformatics prediction, the synonymous mutations Tyr1493Tyr, Gly1678Gly, Ser1756Ser and Pro1960Pro were predicted to cause a frame shift in APC, and the synonymous mutation Tyr486Tyr ($APC^{\Delta 486s}$) was predicted to induce exon skipping in APC (Table III). No intron substitutions in APC or MUTYH were predicted to be disease-associated polymorphisms.

Synonymous variant $APC^{\Delta 486s}$ induces APC exon 12 skipping in a mini-gene splicing assay. Due to the splicing impact predicted by bioinformatics analysis, $APC^{\Delta 486s}$ was further studied to verify its exon splicing impact on the APC gene. Mini-gene systems encoding wild-type and mutant $APC^{\Delta 486s}$ in APC exon 12 were synthesized and inserted into the pEGFP-N1 plasmid. The recombined constructs were subsequently transfected into HeLa cells, and the corresponding PCR products were separated by electrophoresis. The results revealed that the PCR product from the mutant mini-gene was smaller than that from the wild-type mini-gene (Fig. 3). Sequencing results of the two PCR products further confirmed the skipping of exon 12 in APC (data not shown). These results indicated that the mini-gene system encoding mutant $APC^{\Delta 486s}$ induced the skipping of exon 12 in APC.

Discussion

FAP has been linked to germline mutations in the APC gene. However, a number of patients with FAP have no APC mutations (1-3). Among the known genes, evidence has also implicated MUTYH mutations in the pathogenesis of FAP, which may explain up to 25% of all APC mutation-negative FAP cases (7-10). However, a large subset of families with FAP have undetectable pathogenic alterations in APC and MUTYH, and this condition is designated as APC/MUTYH mutation-negative FAP.

This apparent mutation negativity may suggest that APC has alterations that escape detection by routine techniques,

Table III. Computational predictions of the effect on splicing of variants in each gene.

A, APC

Variant	Functionalclass	ESE Finder	ESR Search	PESX	RESCUE_ ESE	Splicingeffect
c.1458T>C(Tyr486Tyr)	Synonymousmutation	Changed	Changed	Changed	Notchanged	Exonskipping
c.4425G>A(Tyr1493Tyr)	Synonymousmutation	Changed	Changed	Changed	Notchanged	Frameshifting
c.5034G>A(Gly1678Gly)	Synonymousmutation	Changed	Changed	Changed	Changed	Frameshifting
c.5268T>G(Ser1756Ser)	Synonymousmutation	Changed	Changed	Changed	Changed	Frameshifting
c.5880G>A(Pro1960Pro)	Synonymousmutation	Changed	Changed	Changed	Changed	Frameshifting
c.220+35T>A	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.645+129A>C	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.1548+133C>A	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.8532+99T>A	Intronmutation	N/A	N/A	N/A	N/A	Noeffect

B, MUTYH

Variant	Functionalclass	ESE Finder	ESR Search	PESX	RESCUE_ ESE	Splicingeffect
c.423G>A(Glu141Glu)	Synonymousmutation	N/A	N/A	N/A	N/A	Noeffect
c.450C>A(Glu150Glu)	Synonymousmutation	N/A	N/A	N/A	N/A	Noeffect
c.1347G>C(Tyr449Tyr)	Synonymousmutation	N/A	N/A	N/A	N/A	Noeffect
c.264+11G>A	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.304+23G>A	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.420+35A>G	Intronmutation	N/A	N/A	N/A	N/A	Noeffect
c.1566+33_ 1566+35het_delTGT	Intronmutation	N/A	N/A	N/A	N/A	Noeffect

APC, adenomatous polyposis coli; MUTYH, mutY DNA glycosylase; ESE, exonic splicing enhancer; ESR, exonic-splicing regulatory; PESX, putative exonic splicing enhancers.

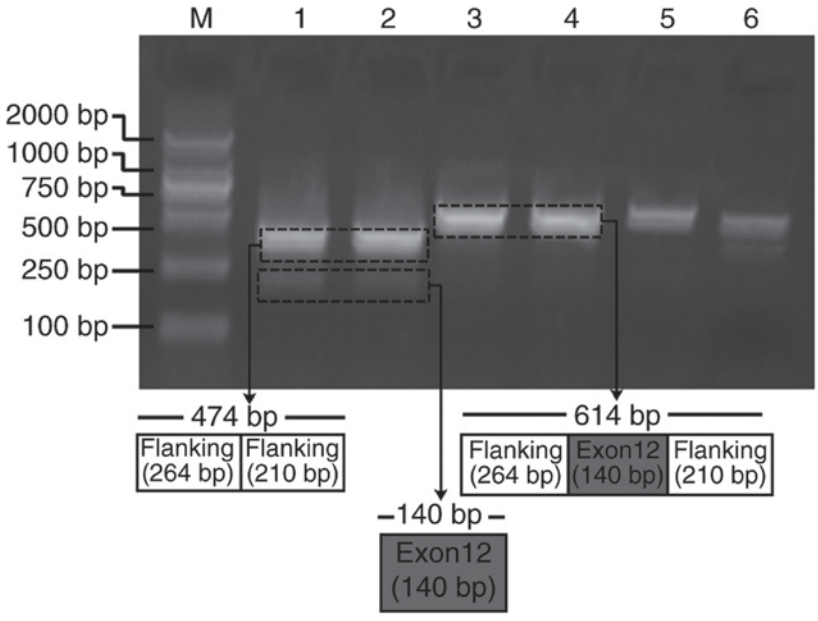

Figure 3. Hybrid mini-gene assay results of the mini-gene system encoding wild-type and mutant APC$^{\Delta 486s}$ in APC exon 12. Lane M indicates the DL2000 molecular marker ladder. Lanes 1 and 2 contain the electrophoresis results of the mutant-type mini-gene system encoding c.1458C. Lanes 3 and 4 contain the electrophoresis results of wild-type mini-gene system encoding c.1458T. APC, adenomatous polyposis coli; bp, base pairs.

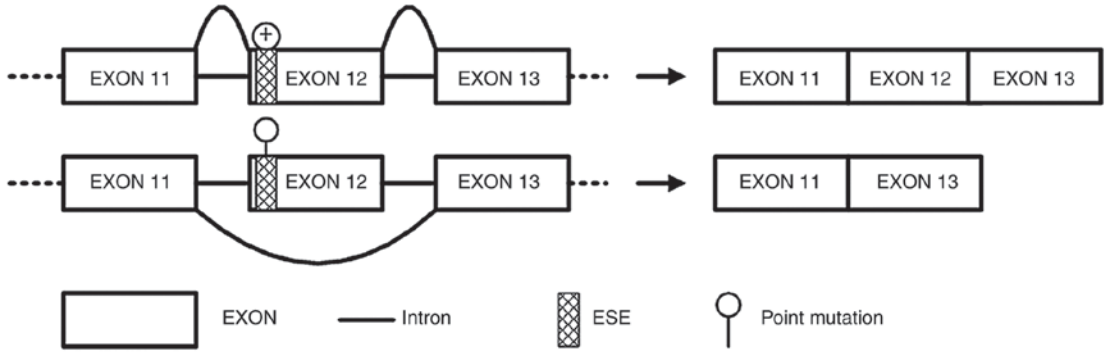

Figure 4. Exon skipping impact induced by APC$^{\Delta 486s}$. Synonymous mutation of c.1458T>C induced a major splicing defect with skipping of exon 12 in APC, and the truncated APC protein results in FAP. APC, adenomatous polyposis coli; ESE, exon splicing enhancer; FAP, familial adenomatous polyposis.

and that other known or unknown genes are involved in FAP predisposition (8). Genetic counselling for these cases of FAP without an identified pathogenic alteration is frequently limited by a lack of knowledge about the pathogenic role of a large fraction of germline sequence variations (34).

In the present study, in order to investigate the germline mutations in Chinese patients with FAP, 13 patients with a classical clinical phenotype were examined by exon-specific DNA sequencing of APC and MUTYH to determine their micromutation types. Following the use of conventional analysis methods, only six types of clearly pathogenic mutations were identified in six individuals. Among the APC/MUTYH mutation-negative cases, seven missense mutations, five synonymous substitutions and three intron mutations were identified in APC, and four missense mutations, three synonymous substitutions and four intron mutations were identified in MUTYH.

Previous reports have indicated that certain missense mutations are deleterious (35). However, examining candidate missense mutations that may cause pathogenic alterations and identifying their relative effects on function is a time-consuming and labor-wasting process. Recently, computational prediction has been proposed as an efficient and economical strategy to screen for potential pathogenic missense mutations (36). By applying bioinformatics tools and databases in the present study, it was predicted that no identified missense mutations in APC or MUTYH were deleterious.

It was previously thought that all synonymous mutations were silent. However, further research into these mutations has demonstrated that silent mutations may alter protein expression, conformation and function (14). In 2007, it was demonstrated that synonymous mutations in transporter ATP binding cassette subfamily B member 1 may be implicated in drug resistance to chemotherapeutic agents, and it was further confirmed that synonymous SNPs (sSNPs) affect protein conformation and have functional and clinical consequences (37). Since then, a number of other synonymous mutations associated with human disease have been reported. For example, polymorphisms rs709816 and rs1061302 in gene NBN may be linked to smoking-associated cancer (38), and polymorphism rs11615 in ERCC1 aids in clinical outcome prediction following oxaliplatin-based chemotherapy in metastatic colorectal cancer (39). In addition, sSNPs rs2229069 and rs2227985 of the gene ABL1in the fusion protein BCR, RhoGEF and GTPase activating protein/ABL proto-oncogene 1, non-receptor tyrosine kinase, may contribute to primary, although not secondary, resistance to tyrosine kinase inhibitors (40). Furthermore, rs2293347 in gene EGFR may be a potential predictor of clinical outcome in patients with advanced non-small cell lung carcinoma when treated with gefitinib (41), and rs1045642 in gene ABCB1 has been associated with multidrug resistance to cancer chemotherapy (42).

Based on the above findings, the identified synonymous and intronic substitutions of APC and MUTYH were further analyzed by applying bioinformatics tools and databases, and the synonymous mutation Tyr486Tyr (APC$^{\Delta 486s}$) was predicted to have induced exon skipping in APC, which suggested that it may have caused pathogenic alterations that lead to FAP predisposition. Therefore, the synonymous substitution c.1458T>C was further studied to investigate its effect on exon splicing of the APC gene. A mini-gene system encoding wild-type or mutant c.1458T>C APC in exon 12 was synthesized and inserted into pEGFP-N1 plasmids. The synonymous substitution of APC$^{\Delta 486s}$ was confirmed to induce a major splicing defect with skipping of exon 12 in APC.

In the present study, DNA sequencing of the APC and MUTYH genes in 13 patients with FAP was conducted. A total of four known pathogenic mutations in APC and two novel disease-associated pathogenic nonsense mutations in MUTYH were identified. For samples that did not present with obvious pathogenic alterations, the functional effects of the identified missense, synonymous and intronic mutations were analyzed with bioinformatics tools and databases. Bioinformatics analyses predicted that the synonymous mutation APC$^{\Delta 486s}$ was likely a disease-causing polymorphism and that it may have induced exon skipping in APC. Using a hybrid mini-gene assay, synonymous SNP APC$^{\Delta 486s}$ was demonstrated to induce a major splicing defect with the skipping of exon 12 in APC (Fig. 4).

Therefore, it was concluded that the synonymous mutation APC$^{\Delta 486s}$ that affected exon splicing in APC was a pathogenic alteration that predisposed patients to APC/MUTYH mutation-negative FAP. However, a limitation to the present study was that the exon skipping effect of synonymous mutation of APC$^{\Delta 486s}$ was predicted by bioinformatics analysis. This was the main focus of the present study; therefore, further

ESE-dependent experiments and protein truncation tests are required to investigate the mechanism of exon skipping and to verify that this occurs in patients with FAP presenting with $APC^{\Delta 486s}$.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

WQL, JY and JD conceived the present study. WQL, JY and YP carried out the bioinformatics prediction work and analyzed the data. WQL, JY, WLL and JD performed the experiments of hybrid mini-gene assay. All authors discussed the results and contributed to the final manuscript.

Ethics approval and consent to participate

The present study was approved by and performed in accordance with the Research Ethics Board of Kunming Medical University (Kunming, China). All participants provided written informed consent, and the ethics committees approved the consent procedure.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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