Identification of a novel mutation associated with familial adenomatous polyposis and colorectal cancer

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Received March 11, 2015; Accepted July 13, 2015

DOI: 10.3892/ijmm.2015.2303

Abstract. Colorectal cancer (CRC) is among the most fatal forms of solid tumor in men and women. While the majority of diagnosed CRC cases are sporadic, 15-25% of patients have a family history of adenomatous polyposis and CRC; however, the associated gene mutations remain largely unidentified. The aim of the present study was to investigate the genomes of a four-generational Chinese Han family with familial adenomatous polyposis and CRC to identify the potential genetic anomalies associated with the disease. Diagnoses were made by physical and enteroscopic examinations of all the family members. Mutational analyses of the potential CRC-associated genes were carried out by direct gene sequencing, and the statistically significant differences in polymorphisms between normal and diseased populations were determined. Multiple sequence alignment and protein modeling were conducted using the Vector NTI and DNAMAN software tools. Clinical and pathological features of all the examined patients were consistent with typical familial adenomatous polyposis (FAP) syndrome. From the genomes of these family members, a 131564T>C (p.1125Val>Ala) mutation was identified in exon 15 of the *APC* gene, and a 1126G>C (p.324Gln>His) mutation was identified in exon 12 of the *MUTYH* gene. The 131564T>C mutation co-segregated with the affected individuals in the family and was specifically associated with the incidence of CRC (P=0.018<0.05). The 1125Val residue was highly conserved in the APC protein, and the p.1125Val>Ala mutation led to changes in the secondary structure and hydrophilicity of the APC protein. In conclusion, the *APC* gene mutation 131564T>C is associated with FAP and the pathogenesis of CRC.

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Key words: colorectal cancer, familial adenomatous polyposis, *APC*, *MUTYH*, environmental risk factors

Introduction

Colorectal cancer (CRC) is among the most fatal forms of solid tumor in men and women worldwide (1), with over 96,000 new cases of colon cancer and 40,000 new cases of rectal cancer diagnosed annually in the US (2). While the majority of CRC cases are sporadic, 15-25% of patients have a family history (3,4), and 5% are diagnosed with inherited CRC syndrome (5). A number of genes have been implicated in the pathogenesis of CRC, such as tumor-suppressor genes (*APC*, *TP53* and *CDKN2A*), proto-oncogenes (*KRAS* and *HRAS*) and DNA repair genes (*MUTYH*) (6); however, specific mutations in these genes have not been identified in numerous CRC patients.

Hereditary colorectal polyposis includes a range of disorders passed on through autosomal dominant inheritance, and is divided into Lynch syndrome and familial adenomatous polyposis (FAP) (7). Lynch syndrome is characterized by the absence of polyposis (8), a positive family history and high risk for developing CRC, and a predisposition for extracolonic malignancies (such as endometrial, ovarian and gastric carcinomas) (9). Patients suspected of having Lynch syndrome are first tested for germline mutations in the mismatch repair gene. Patients without polyposis or polyposis family history, and not presenting Lynch syndrome should undergo genetic counseling to determine the cancer risk (1).

Polyposis syndrome is one of the most common syndromes associated with familial CRC, and is involved with a number of diseases, including FAP, mutY Homolog (E. coli) (MUTYH)-associated polyposis (MAP), Peutz-Jeghers syndrome and juvenile polyposis (10,11). However, the majority

of the familial CRC cases do not present polyposis, and potentially associated gene mutations are largely unknown (12). Adenomatous polyposis coli (APC), a key regulator of β-catenin in the Wnt/β-catenin signaling pathway, has a critical role in several fundamental cell processes, including cell division and signal transduction, particularly in tumor suppression (13,14). Several mutations and deletions, as well as promoter methylation, have been identified in the *APC* gene (15). Of significance, a number of those genetic or epigenetic changes have also been described in FAP syndrome, and more than two-thirds of CRC and adenomas have somatic mutations in the *APC* gene (16,17). In addition to CRC, mutations of *APC* have been reported in other tumors, including cancers in the liver (4), stomach (5-7), lung (8), breast (9) and the brain (cerebellar medulloblastoma) (10). However, the role of these types of mutations in the development of tumors has not been fully elucidated (11-13). The present study reports a novel mutation on exon 15 of the *APC* gene in four generations of a Chinese family with FAP and 200 sporadic cases of adenomatous polyposis, and furthermore, suggests a potential mechanism by which this mutation contributes to the pathogenesis of CRC.

Materials and methods

Study population and DNA collection. The members of a four-generational Chinese Han family with familial adenomatous polyposis (FAP) (Fig. 1), 200 sporadic adenomatous polyposis cases and 220 normal controls (Table I) were included in this study, which was conducted at the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Written informed consent was obtained from each participant (or guardian for all the participants <18 years of age) and the study was reviewed and approved by the Ethics Committee of Harbin Medical University, consistent with the 1975 Declaration of Helsinki. The medical history was recorded in detail for all the enrolled participants. Each patient received physical and enteroscopic examinations. Genomic DNA was extracted from peripheral blood leukocytes of each participant using standard protocols.

DNA analysis. The exons and splicing sites of the *APC* and *MUTYH* genes were amplified by polymerase chain reaction (PCR) with the primers (data not shown), and the PCR products were sequenced using standard protocols (18) for mutational analysis.

Statistical analysis of disease-associated polymorphisms. Relevant polymorphisms were determined by DNA sequencing for all the family members. The prevalence of these polymorphisms among 200 sporadic adenomatous polyposis cases and 220 normal controls was subsequently analyzed. Statistical analyses were performed using χ^2 tests to calculate the odds ratios and P-values using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA).

Multiple sequence alignment and analysis of protein models. From the NCBI website (http://www.ncbi.nlm.nih.gov/), the APC and MUTYH protein sequences of various species were obtained and multiple-sequence alignments of the proteins were conducted using Vector NTI software (Life Technologies, Grand Island, NY, USA). The protein structures of the mutant and wild-type proteins were predicted and analyzed by Swiss-model software (version 3.5) (19-22), and DNAMAN software (Lynnon Corp., Quebec, Canada).

Table I. Clinical characteristics of the population used for polymorphism-association analyses.

Parameter	Sporadic adenomatous polyposis	Control
Sample, n	200	220
Male/female, n	120/80	105/115
Age, years	58.65±12.13	59.36±4.21

Results

Clinical characteristics. The proband was a 32-year-old male (II:5; Fig. 1), admitted for diarrhea and weight reduction over the course of one year. The medical history of the patient revealed similar clinical features for ~5 years, however, the symptoms were treated without systematic examination for a diagnosis. Physical examination showed that the abdomen of the patient was slightly distended and nontender. Blood analysis showed the presence of a significant anemia; however, there was no bleeding or clots on rectal examination. Colonoscopy revealed numerous polyps (>100) measuring between 0.2-2 cm in diameter along the colon and rectum, confirmed as tubular adenoma with low-grade dysplasia. A mass was observed in the transverse colon measuring 3.0x3.0 cm. Biopsy of the polyp revealed typical adenomatous polyp features, and biopsy of the tumor demonstrated moderately differentiated adenocarcinoma. Endoscopic examination of the upper gastrointestinal system showed multiple gastric polyps in the fundus and upper body. Pathological examination confirmed fundic gland polyps. A computed tomographic scan also revealed tumors in the transverse colon; however, there was no evidence of liver or lymph node metastases.

In this four-generation family, there were 7 affected individuals with FAP syndrome that showed numerous polyps along the colon and rectum (Fig. 2); 1 affected member with endometrial cancer, 3 with the *APC* mutation but no corresponding clinical features, and 42 unaffected individuals (Table II). All the diagnoses were confirmed by three colorectal cancer specialists. There was no history of other systemic abnormalities in the family.

DNA analysis. Sanger sequencing of the amplified fragments in two affected family members identified a single base alteration, 131564T>C (Fig. 3), in exon 15 of the *APC* gene (GI:324) located at 5q21-q22, resulting in the substitution of Val to Ala at codon 1125 (p.1125Val>Ala), and a single base alteration, 1126G>C (Fig. 3) in exon 12 of the *MUTYH* gene (GI:4595) located at 1p34.1, resulting in the substitution of Gln to His at codon 324 (p.324Gln>His). The remaining coding sequence of the two genes showed no other changes.

Further sequence analysis revealed that the 131564T>C alteration in the *APC* gene was co-segregated with all the affected individuals in the family (except III:4, 21 and 25; analyzed in the discussion). However, the 1126G>C alteration

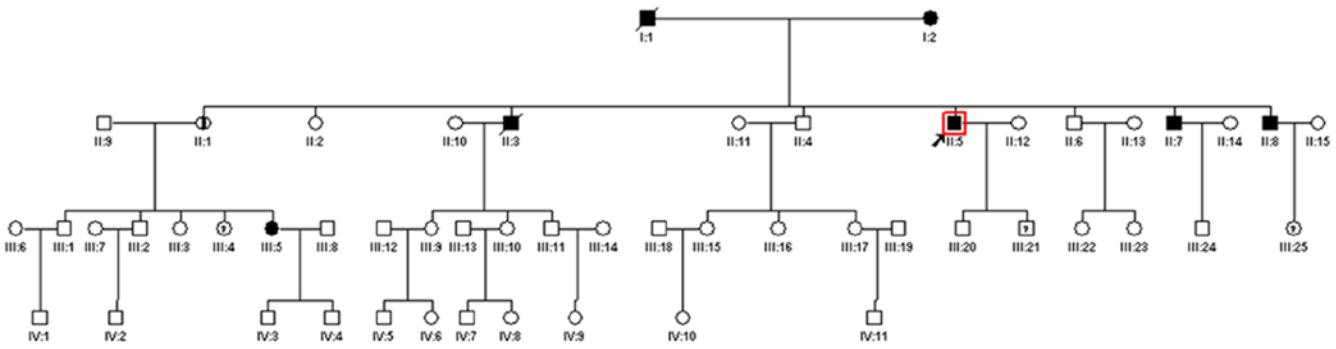

Figure 1. Pedigree of a four-generational Chinese Han family afflicted with familial adenomatous polyposis (FAP). Squares and circles indicate males and females, respectively. Filled symbols denote a diseased status. Empty symbols with a question mark inside denote mutation carriers with no clinical features. Normal individuals are shown as empty symbols.

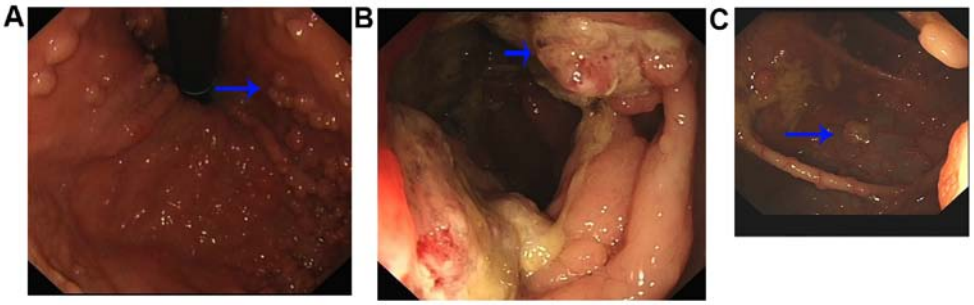

Figure 2. Enteroscopic examination of the proband individual (II:5). Images of the affected individual II:5 showed numerous polyps (arrows) measuring between 0.2-2 cm in diameter along the colon and rectum, confirmed as tubular adenoma with low-grade dysplasia. Endoscopic examination of the upper gastrointestinal system showed multiple gastric polyps in the fundus and upper body. (A) Numerous polyps (arrow) along the colon and rectum; (B) tubular adenoma (arrow) with low grade dysplasia; (C) Polyps (arrow) measured about 2 cm in diameter. There was no evidence of liver or lymph node metastases.

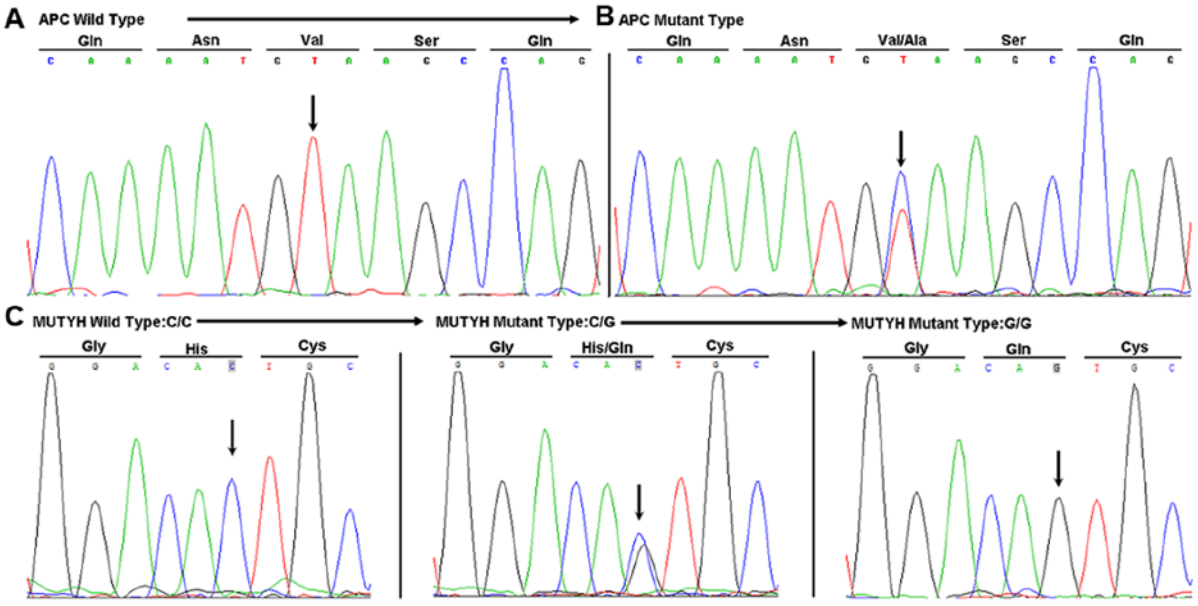

Figure 3. DNA sequence chromatogram of the 131564T>C mutation in *APC* and 1126G>C variation in *MUTYH*. (A) A representative DNA sequence chromatogram of the unaffected family members; 220 normal controls and 195 patients with sporadic adenomatous polyposis. (B) A representative DNA sequence chromatogram of the affected family members, III:4, III:21 and III:25, and 5 sporadic adenomatous polyposis patients. (C) DNA sequence chromatogram of the three polymorphisms, G/G, G/C and C/C, identified in the *MUTYH* gene in all family members, and the population used for disease-association analyses.

in the *MUTYH* gene was not co-segregated with any affected individuals in the family (data not shown).

Statistical analysis of the polymorphisms associated with disease. To further test any possible associations between the genetic

Table II. Clinical features and gene polymorphism of the affected individuals in the family.

Patient identity	Gender	Age, years	Onset age, years	Risk factor	Onset symptoms	Location	Age at death, years	CEA (ng/ml)	CA199 (U/ml)	TNM stage	Pathological type	*APC* 131564T>C	*MUTYH* 1126G>C
I:1	M	84	82	Smoking	Bowel obstruction	Caecum	84	2.13	4.65	IIIa	Adenocarcinoma	/	/
I:2	F	79	75	Smoking	Hematochezia	Rectum	/	5.34	3.21	No	Adenocarcinoma	T/C	G/C
II:1	M	58	54	No	Vaginal bleeding	Uterus	/	/	/	IIa	Endometrioid carcinoma	T/C	G/G
II:3	M	55	50	Smoking	Abdominal pain	Rectum	55	4.31	10.2	IIIb	Mucinous adenocarcinoma	/	/
II:5	M	52	48	Smoking	Abdominal pain	Ascending colon	52	6.45	5.75	/	Adenocarcinoma	T/C	G/C
II:7	M	39	22	Smoking	Abdominal pain	Sigmoid colon	/	3.46	2.47	/	Intraepithelial neoplasia	T/C	G/G
II:8	M	30	29	Smoking	Anemia	Caecum	/	3.42	3.21	IIIa	Adenocarcinoma	T/C	G/C
III:4	F	25	No	No	/	/	/	/	/	/	/	T/C	G/C
III:5	F	30	29	Smoking	Hematochezia	Rectum	/	3.45	5.87	IIb	Adenocarcinoma	T/C	G/C
III:21	M	24	No	No	/	/	/	/	/	/	/	T/C	G/G
III:25	F	8	No	No	/	/	/	/	/	/	/	T/C	G/C

CEA, carcinoembryonic antigen; CA199, carbohydrate antigen 19-9; TNM, tumor-node-metastasis.

Table III. Genotype and allele frequency of the 131564T>C mutation and 1126G>C variation in 200 Chinese Han sporadic adenomatous polyposis patients and 220 non-CRC controls.

Gene/variation	SAP, frequency (%)	Control, frequency (%)
MUTYH/1126G<C		
Genotype		
G/G	75 (37.5)	81 (36.8)
G/C	105 (52.5)	104 (47.3)
C/C	20 (10.0)	35 (15.9)
Allele		
G	255 (63.8)	266 (60.5)
C	145 (36.3)	174 (39.5)
APC/131564T>C		
Genotype		
T/T	195 (97.5)	220 (100.0)
T/C	5 (2.5)	0 (0.0)
C/C	0 (0.0)	0 (0.0)
Allele		
T	395 (98.8)	440 (100.0)
C	5 (1.3)	0 (0.0)

CRC, colorectal cancer; SAP, sporadic adenomatous polyposis.

mutations and CRC, polymorphism-association analyses were conducted and the 131564T>C variation in the *APC* gene was clearly associated with the risk of CRC (P=0.018<0.05); however, there was no statistical significance between the 1126G<C variation in the *MUTYH* gene and CRC (Tables III and IV). The Hardy-Weinberg equilibrium test was also conducted for the CRC patients and the control population, and identified that they were in line with the Hardy-Weinberg equilibrium.

Evolutionary conservation of protein across the species. APC and MUTYH protein sequences were compared across multiple species including birds, fish, rodents and primates. Multiple-sequence alignment analysis showed that with the exception of *Saccoglossus kowalevskii*, *Danio rerio* and *Xenopus laevis*, the 1125Val residue in the APC protein was highly conserved, but the 324Gln residue in the MUTYH protein was only conserved in rodents and primates (Fig. 4).

3D modeling of protein structure. The secondary structures, hydrophobicity and hydrophilicity of the wild and mutant proteins were also compared. The online bioinformatics Swiss-model software (version 3.5) was used to predict the wild-type and mutant APC protein structure. The p.1125Val>Ala mutation made minor changes in ~10 amino acids within the secondary structure. For hydrophilicity, ~700 local amino acids were changed in the mutant APC protein; however, the hydrophobicity exhibited fewer changes (data not shown). The p.1125Val>Ala mutation also exerted a minor effect on the tertiary structure of the protein (Fig. 5).

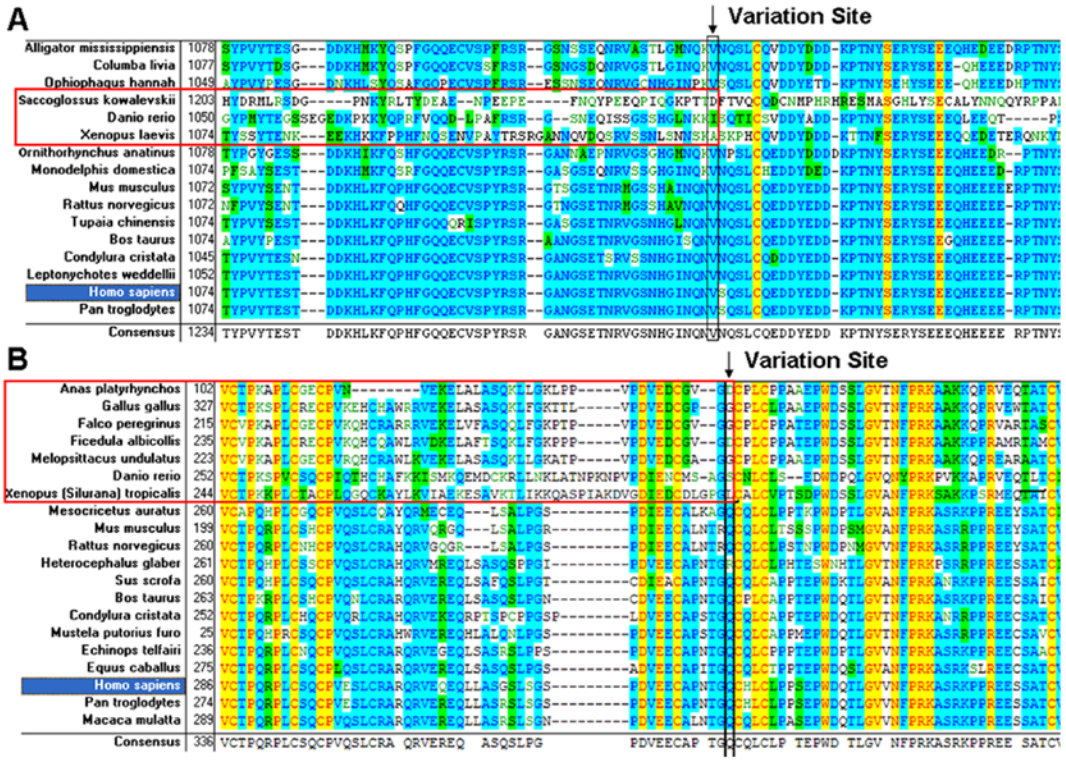

Figure 4. Analysis of protein sequences across species. (A) Multiple-sequence alignment of the adenomatous polyposis coli (APC) protein family. (B) Multiple-sequence alignment of the mutY Homolog (E. coli) (MUTYH) protein family. The 1125Val residue in APC was more highly conserved compared to the 324Gln residue in the MUTYH protein.

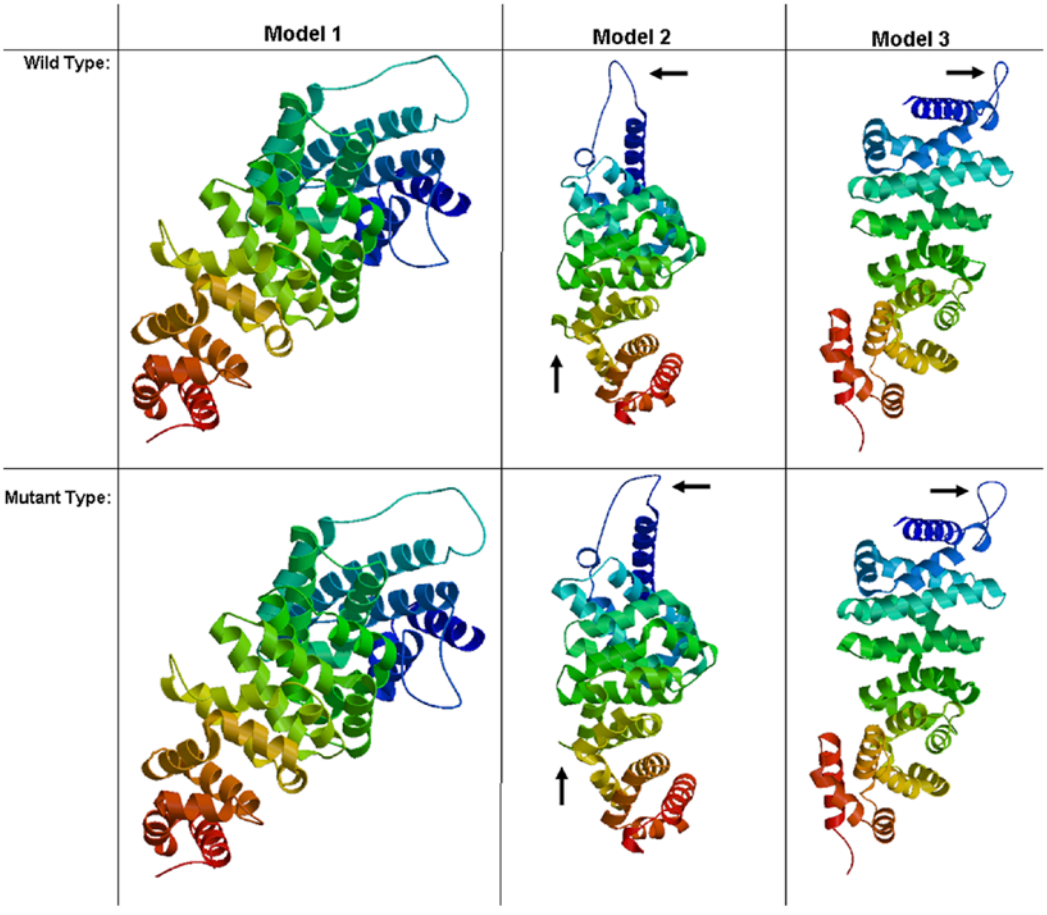

Figure 5. Comparison of wild-type and mutant adenomatous polyposis coli protein tertiary structure. The p.1125Val>Ala mutation exerted minor changes on the space of the peptide chain of the tertiary structure of the protein (arrows).

Table IV. Associations with the risk of sporadic adenomatous polyposis in the Chinese populations with the 131564T>C mutation within *APC*, but not the 1126G>C variation in *MUTYH*.

		Pearson χ^2				Pearson's R			
Gene/variation	Type	Value	Min. count[a]	df	Asymp. sig. (2-sided)	Value	Asymp. std. error[b]	Approx. T[c]	Approx. sig.
MUTYH/1126G<C	Genotype	3.382[a]	26.19	2	0.184	0.049	0.048	1.011	0.313[d]
	Allele	0.966[a]	151.90	1	0.326	0.034	0.034	0.982	0.326[d]
APC/131564T>C	Genotype	5.566[a]	2.38	1	0.018	-0.115	0.026	-2.369	0.018[d]
	Allele	5.533[a]	2.38	1	0.019	-0.081	0.018	-2.357	0.019[d]

[a]Minimum expected count; [b]not assuming the null hypothesis; [c]using the asymptotic standard error assuming the null hypothesis; [d]based on normal approximation.

The degree of change caused by the p.324Gln>His variation in the MUTYH protein was less than the changed caused by the p.1125Val>Ala variation to the APC protein (data not shown).

Discussion

Familial adenomatous polyposis (FAP) is a disease of autosomal dominant inheritance, with the main clinical manifestations consisting of multiple adenomatous polyps formed in the colon and rectum of affected patients (23). Approximately 80% of sporadic colorectal tumors are caused by mutations in the *APC* gene (24), and recently, two non-conservative mutations, Y165C and G382D, were identified in the *MUTYH* gene (25). The *MUTYH* gene encodes a DNA glycosylase that is involved in the repair of oxidative DNA damage. These *MUTYH* gene mutations were shown to cause an increased tendency of somatic CG→AT transversion in the *APC* gene in several colorectal adenoma or carcinoma patients (26). Further research revealed that 7-10% of FAP patients exhibit *MUTYH* mutations (27,28), several of which can enhance the spontaneous mutator phenotype resulting in the accumulation of 8-oxoguanine (oxoG) DNA in response to oxidative stress (29). These two MUTYH mutations, Y165C and G382D, have been shown to reduce the activity of mutY in removing A from G:A mismatches in *E. coli*, and have also demonstrated a decrease in activity of the human *MUTYH* enzyme in the excision of A opposite 8-oxoG, which ultimately led to the formation of tumors (30,31). This suggests that the *MUTYH* gene has an important role in the development of FAP, *APC*-associated colorectal tumors and the generation of *APC* mutations.

In the present study, a c.131564T>C (p.Val1125Ala) mutation in the *APC* gene was identified, which was co-segregated with disease in a multigenerational family afflicted with FAP. This mutation was not found in unaffected family members or in 220 random subjects selected from a normal population that served as a control group. Notably, 5 patients out of 200 with sporadic adenomatous polyposis also had the p.Val1125Ala mutation. Taken together, the frequency of this *APC* mutation was high in the sporadic adenomatous polyposis population, and the mutation was possibly the main cause of disease in the family studied.

The way in which one amino acid substitution in the APC protein can lead to the phenotypic changes observed in FAP is not fully understood. The present results demonstrate that the p.1125Val>Ala mutation results in minor changes in the secondary and tertiary structure, as well as hydrophilicity. The region of the APC protein from codon 1265 to 2035 is the binding site for β-catenin, and is essential for β-catenin degradation (32,33). Mutations in this functional domain can cause changes in β-catenin binding, which is believed to have an important role in the pathogenesis of FAP (34). FAP patients with mutations in this subunit (particularly beyond codon 1309 or 1444) are more likely to develop desmoid disease, which is more severe than patients with mutations in other regions of the *APC* gene (33,35). Around 21% of patients with desmoid tumors had *APC* mutations downstream of the 1444 codon, and similar mutations were identified in only 4.1% of patients with FAP (33). In the present study, the mutation identified in the Chinese Han family was in the 1125 codon (downstream of the 1444 codon), and the main clinical features of the patients were adenocarcinoma and numerous polyps along the colon and rectum. Therefore, this suggests that the 1125 codon is not as important for the function of the APC protein as codon 1265-2035, which is the binding site for β-catenin (32,33).

A c.1126G>C (p.324Gln>His) variation in the *MUTYH* gene was also identified. The majority of CRC patients with *MUTYH* mutations exhibit fewer polyps, and in certain cases have no polyps; and only extremely few cases have >500 polyps (23). However, certain *MUTYH* mutation CRC patients may develop secondary cancers in the skin, ovary, bladder and breast. In the present study, one patient in the family had endometrial cancer; however, the majority presented with adenocarcinoma and numerous polyps (>100) along the colon and rectum. Furthermore, the c.1126G>C (p.324Gln>His) variation in the *MUTYH* gene was not co-segregated with the studied family, and was also not associated with sporadic adenomatous polyposis disease. Taken together, the effects of the c.1126G>C (p.324Gln>His) mutation in the *MUTYH* gene are negligible with regards to cancer development in this family.

The present clinical investigation showed that the affected family members with the T/C-*APC* mutation were either of relatively old age or were reported smokers. The

average age of onset of the disease is 48 years, ranging from 21 to 70 years (36). Notably, certain younger *APC*-mutation carriers have no clinical features of the disease, suggesting that age may be a potential risk factor for FAP. While certain risk factors can increase the chance of developing colorectal polyps or colorectal cancer, such as increased age (37) and smoking (38), the development of CRC from adenomatous polyps can be prevented through intensive colorectal screening (39).

In conclusion, a Chinese FAP family with adenocarcinoma and numerous polyps along the colon and rectum, in which several gene variants that may have a role in the pathogenesis of this disease were identified, was reported. The c.131564T>C (p.Val1125Ala) mutation in the *APC* gene was co-segregated with the patients with the disease in this family, and was also directly associated with sporadic adenomatous polyposis disease.

Acknowledgements

The authors would like to thank the patients and the family members for their cooperation and participation in the present study. The study was supported by a grant from the Heilongjiang Innovation Research Foundation for Graduate Studies (no. YJSCX2014-10HYD); grants of the National Natural Science Foundation of China (nos. NSFC81271786, 81110378, 30970119, 81030029 and 81272706); a grant from the Natural Science Foundation of Heilongjiang Province (no. QC2013C086); and a grant from the Science Foundation of Health Department of Heilongjiang Province (no. 2012-452).

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