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Mutation analysis of the *MYH* gene in unrelated Czech *APC* mutation-negative polyposis patients

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ABSTRACT

Some of the *APC* negative FAP and AFAP cases have recently been found to be attributable to *MYH* associated polyposis (MAP). MAP is an autosomal recessive syndrome associated with 5–100 colorectal adenomas and caused by mutation in the *MYH* gene. Here, we screened for germline *MYH* mutations in 82 *APC*-mutation-negative probands with classical and attenuated familial adenomatous polyposis using the denaturing high performance liquid chromatography (DHPLC) method in combination with sequencing. Altogether 12 previously reported changes and four novel genetic alterations, mostly in intronic sequences, were identified. The results revealed the presence of biallelic germline *MYH* mutations in two patients. These patients were compound heterozygotes for two of the most common germline mutations c.494 A > G (p.Y165C); c.1145 G > A (p.G382D). These variants are established to be associated with adenomatous polyposis and colorectal cancer. No novel pathogenic mutation has been identified in our study.

1. Introduction

Familial adenomatous polyposis (FAP, OMIM 17500) is a hereditary predisposition to colorectal cancer (CRC) characterised by the development of hundreds to thousands of adenomatous polyps in the colon and rectum. The autosomal dominant inherited FAP is associated with germline mutations in the *APC* (adenomatous polyposis coli) tumour-suppressor gene, which encodes a protein that plays a critical role in the regulation of colonic cell growth. Patients with FAP may also develop extra-colonic manifestations of the disorder, including skin and bone cysts, gastric and duodenal adenomas, desmoid tumours and congenital hypertrophy of the retinal pigment epithelium (CHRPE).[1,2] An attenuated form of disease (AFAP - attenuated familial adenomatous polyposis) is associated with smaller numbers (<100) of colorectal adenomas and later clinical presentation. AFAP is caused by mutations in the most 5′or 3′ regions of *APC* or in the alternative spliced region of exon 9.[1,3,4] In 20–30% of patients with FAP and up to 90% of those with AFAP, no pathogenic germline mutation in the *APC* gene can be identified.[5] Some of the *APC* negative FAP and AFAP cases have recently been found to be attributable to *MYH* associated polyposis (MAP, OMIM 604933). MAP is an autosomal recessive syndrome associated

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with 5–100 colorectal adenomas and caused by germline mutations in the *MYH* gene.[6–8]

The *MYH* gene is located on chromosome 1p34.3-p32.1 and consists of 16 exons. MYH protein takes part in the base excision repair (BER) pathway, which plays a basic role in the repair of mutations caused by reactive oxygen species generated during the aerobic metabolism. The most stable product of oxidative damage is the guanine aduct 8-oxo-7,8-dihydroxy-2′-deoxyguanosine (8-oxoG), which tends to mispair with adenine, leading to G:C > T:A transversion after the next replication on other genes, including *APC*.[6,9] The critical steps in DNA repairing by BER are carried out by a set of genes: *MTH*, *OGG1*, *MYH*. The products of these genes act synergistically to prevent mutagenesis induced by 8-oxoG.[10] No germline mutation has been detected in the *MTH* and *OGG1*.[6,7,11]

2. Materials and methods

A set of 82 affected individuals from the Czech Republic was screened for genetic variants in the *MYH* gene. Germline *MYH* mutations were screened in 25 patients with multiple (three to 100) colorectal adenomas, in 28 probands with classical familial adenomatous polyposis (>100 adenomas) and in 29 patients with suspected FAP. All of them came from various health institutions in Czech Republic and were referred on the basis of colonoscopic findings and/or positive family history. Phenotype classification was based on the following criteria: the number of polyps and the age at diagnosis of FAP. The phenotype was classified as typical FAP when the patient presented with >100 polyps in the second and/or third decade of life with the occurrence of CRC in or before the fourth decade of life. The multiple colorectal adenomas phenotype was characterised by the occurrence of <100 of polyps in the third or fourth decade of life and later occurrence of the CRC. Twenty-three of the patients with FAP and ten patients with multiple colorectal adenomas phenotype displayed a family history compatible with an autosomal dominant pattern of inheritance (that is, more than one generation with either polyposis or CRC); in the reminder there was no detailed family history available. Patients with suspected FAP represent the group in which clinical information was not sufficiently supplied and we were not able to categorise them.

In all patients, no germline *APC* mutation was identified by means of denaturing gradient gel electrophoresis, protein truncation test and/or direct sequencing. In addition, 100 controls from the Czech population were enrolled to establish the carrier frequency of selected *MYH* polymorphic variants. The study was approved with the ethics committee and all subjects were given informed consent.

2.1. DNA analysis

Genomic DNA was isolated from peripheral blood lymphocytes using standard salting-out procedures.[12] DNA was amplified by the polymerase chain reaction (PCR).

Mutation screening involves the entire coding region (exon 1–16) and flanking exon-intron boundaries of the *MYH* gene. PCR primers are described in more detail by Al-Tassan.[6] Each 25 μl reaction mixture contained 120–250 ng of genomic DNA, 1.5 mM $MgCl_2$, 100 ng of each primer, 0.1 mM of each dNTP and 0.75 unit of AmpliTaq Gold® Polymerase in the buffer provided by the manufacturer (Applied Biosystems, Foster City, CA, USA). Amplification conditions were 96 °C for 10 min as a denaturation step followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C (exon 3–5, 7–10, 13,15) or at 57 °C (exon 1,2,6,11,12,14,16), 20 s at 72 °C, ending with 3 min at 70 °C.

We established mutation analysis using denaturing high performance liquid chromatography (DHPLC) on WAVE 3500 system (Transgenomic, Omaha, NE, USA) as a pre-screening method to detect DNA sequence changes. Five μl of PCR were injected on the DNASep column and eluted over 5 min by a linear acetonitrile (ACN) gradient of 8% ACN v/v at optimised temperature. The particular DHPLC running conditions for each PCR amplicon (ACN gradient characteristics and column temperature) were designed using the Navigator Software 1.6.4 (Transgenomic) and determined following optimisation prior to *MYH* genotyping in the study (Table 1). Chromatograms were recorded using fluorescence detector (at wavelength of 260 nm) and normalised by a mutation calling routine in Navigator Software.

The samples exhibited aberrant elution profiles of on DHPLC chromatograms were sequenced bi-directionally on automatic fluorescent sequencer ABI Prism® 310 Genetic Analyzer using BigDye Terminator ver. 3.1 (Applied Biosystems).

One hundred control samples were directly genotyped for ten selected polymorphic variants, present at both intronic and exonic sequences. The fragment covering the genomic region from exon 2 to exon 15 of the *MYH* gene was amplified using the long range PCR method with forward primer 5′cattgtgactgactgctttggctgggtc3′ and the reversed primer 5′ggagtggagaatgttcacccagacattcgttag3′. The amplification conditions were performed in the 15 μl reaction mixture containing 120–250 ng of genomic DNA, 1.25 mM $MgCl_2$, 50 ng of each primer, 0.2 mM of each dNTP and 0.15 μl Long PCR Enzyme Mix in the buffer provided by the manufacturer (Fermentas, Vilnius, Lithuania). Amplification conditions involved initial denaturation at 94 °C for 2 min followed by 38 cycles of 20 s at 94 °C, 3 min at 68 °C (with ramping 3 s/cycle), ending with 5 min at 68 °C. The long PCR product was further analysed by ABI Prism® SNaPshot™ Multiplex Kit according to the instructions of the manufacturer (Applied Biosystems).

Publicly available software (GENSCAN, NetGene2 splice site prediction web server) was used to test the potential role of missense and synonymous variants on splicing.

Publicly available software ESEfinder 3.0 (http://rulai.cshl.edu/tools/ESE/) was used to find exonic splicing enhancers and intronic splice site sequences.

2.2. Statistical analysis

Allele frequencies were obtained by allele counting and standard errors were assigned. Each polymorphic locus was tested for significant deviation of genotype distributions from Hardy–Weinberg equilibrium using the χ^2 test ($p < 0.05$; 1d.f.). Significance of allele frequency differences between cases and controls was evaluated using the Yates corrected χ^2 test. A p value of 0.05 was considered statistically significant. These computations were undertaken using the statistical

Table 1 – DHPLC conditions and lengths of PCR amplicons for genotyping of a MYH on WAVE 3500 (several amplicons (covering exons 3, 11, 13) were analysed in two different temperatures)

PCR fragment	Fragment length	Column temperature	Gradient 8% ACN v/v
exon 1	133 bp	66,1 °C	45.9 – 54.9
exon 2	262 bp	59,8 °C	53.5 – 62.5
exon 3	272 bp	61,5 °C	55.8 – 64.8
		62,5 °C	53.8 – 62.8
exon 4	167 bp	62,9 °C	48.6 – 57.6
exon 5	189 bp	62,1 °C	50.2 – 59.2
exon 6	185 bp	60,4 °C	49.9 – 58.9
exon 7	186 bp	61,2 °C	49.9 – 58.9
exon 8	240 bp	64,3 °C	49.6 – 58.6
exon 9	196 bp	62,4 °C	50.5 – 59.5
exon 10	262 bp	63,9 °C	51.5 – 60.5
exon 11	251 bp	61,1 °C	53.1 – 62.1
		62,6 °C	52.1 – 61.1
exon 12	297 bp	62,9 °C	54.6 – 63.6
exon 13	242 bp	64,0 °C	52.7 – 61.7
		65,0 °C	52.7 – 61.7
exon 14	256 bp	59,6 °C	53.3 – 62.3
exon 15	205 bp	58,9 °C	51.1 – 60.1
exon 16	224 bp	61,1 °C	51.9 – 60.9

software STATISTICA, version 7.1 (StatSoft, Inc., USA; www.statsoft.com).

3. Results

We screened the whole coding region of the *MYH* gene for genetic alterations in 82 unrelated Czech patients with clinically diagnosed adenomatous polyposis, in whom no germline *APC* mutation had been detected.

Molecular genetic analysis showed that two patients (patient 70, 79) were carriers of a biallelic mutation in the *MYH*-gene. They were compound heterozygotes for the p.Y165C and p.G382D missense variants. The p.Y165C and p.G382D missense mutations are located in the ENDO3c and NUDIX domains of the protein respectively and their functional role has been previously demonstrated.[6]The patient 79 had an attenuated FAP phenotype and he had no reported family history of colorectal cancer and/or multiple adenomas. He had multiple adenomas/attenuated (<100 polyps) FAP and colorectal cancer at presentation at the age of diagnosis (56 years) and he underwent a total colectomy.[13] The patient 70 had an attenuated FAP phenotype and no reported family history of colorectal cancer and/or multiple adenomas. He had less than 100 polyps at the age of diagnosis (51 years). Their phenotypes closely resembled those described in previous studies[2,7] among patients with biallelic *MYH* mutations.

We also detected a patient with only one *MYH* germline mutation p.Y165C. This patient 197 had less than 100 polyps at the age of diagnosis (71 years) and he had no reported family history of colorectal cancer and/or multiple adenomas.

No patients harboured truncating mutation in the *MYH* gene in our study.

We further identified four previously reported exons' polymorphisms: p.V22M (c.64 *G* > *A*) in exon 2, p.Q324H (c.972 *G* > *C*) in exon 12, p.R412C (c.1234 *C* > *T*) in exon 13 and p.S501F (c.1502 *C* > *T*) in exon 16[6,7,14] (Table 2). These polymorphisms were detected with allele frequencies of 3.05%, 27.44%, 0.61% and 0.61%, respectively. The frequencies of these polymorphisms are consistent with the published data.[6,14,20] We have also detected four previously reported changes in intronic sequences: c.157 + 30 *A* > *G*, c.462 + 35 *G* > *A*, c.648 + 21 *C* > *A*, c.1435 – 40 *G* > *C*, in our study (Table 2). Two rare variants in intron 11 (c.956 – 9 *C* > *T*) and intron 12 (c.1145 – 27 *C* > *T*) were also detected. The frequencies of these two variants (0.61% and 1.83%, respectively) were similar in both patients and controls and were consistent with the published data (Table 3). Four novel sequence changes have been detected (mutations/polymorphisms) in both *MYH* coding and intronic sequences: p.P502P (c.1506 *G* > *A*), c.36 + 11 *C* > *T*, c.347 – 13 *C* > *G*, and c.1435 – 17 *C* > *G*. The silent change p.P502P (c.1506 *G* > *A*) was only found in one of the 82 patients.

The novel variants in intron 1 (c.36 + 11 *C* > *T*), intron 4 (c.347 – 13 *C* > *G*) and intron 14 (c.1435 – 17 *C* > *G*) were detected with allele frequencies of 1.8%, 3.66% and 1.2%, respectively. The variant c.36 + 11 *C* > *T* was not screened in control samples, because we only genotyped polymorphic variants presented at region between exon 2 and exon 15 of the *MYH* gene. Variant c.1435 – 17 *C* > *G* was not detected in control samples.

There were 31 individuals between cases carried two variants in our study. Twelve individuals carried c.462 + 35 *G* > *A* and c.1435 – 40 *G* > *C*, three individuals carried c.157 + 30 *A* > *G* and c.972 *G* > *C*. Sixteen individuals were carriers for another combination of sequence variants.

The genotype frequencies distribution for all polymorphic sites was in Hardy–Weinberg equilibrium in cases as well as in controls.

We have also tested the potential role of missense and synonymous *MYH* variants on splicing by publicly available software GENSCAN and NetGene2 splice site prediction web server. However, only one of the missense and synonymous

Table 2 – Summary of found sequence variants in MYH gene

Nucleotide change	Amino acid change	Position	Gene change character
c.36 + 11 C > T		intron 1	unknown (novel)
c.64 G > A	p.V22M	exon 2	polymorphism[20]
c.157 + 30 A > G		intron 2	unknown[21]
c.347 – 13 C > G		intron 4	unknown (novel)
c.462 + 35 G > A		intron 6	unknown[21]
c.494 A > G	p.Y165C	exon 7	pathogenic mutation[6]
c.648 + 21 C > A		intron 8	unknown[21]
c.956 – 9 C > T		intron 11	unknown[14]
c.972 G > C	p.Q324H	exon 12	polymorphism[6,20]
c.1145 – 27 C > T		intron 12	unknown[19]
c.1145 G > A	p.G382D	exon 13	pathogenic mutation[6]
c.1234 C > T	p.R412C	exon 13	polymorphism[6]
c.1435 – 17 C > G		intron 14	unknown (novel)
c.1435 – 40 G > C		intron 14	unknown[21]
c.1502 C > T	p.S501F	exon 16	polymorphism[6,21]
c.1506 G > A	p.P502P	exon 16	unknown (novel)

Table 3 – Minor allele frequency of MYH gene polymorphisms in cases and controls and the results of statistic analysis of allele frequency differences between cases and controls

Genetic alteration	Frequency ± SD (mutant alleles/total)		p value	Yates corrected χ^2
	Case	Control		
c.157 + 30 A > G (intron 2)	0.0305 ± 0.013 (5/164)	0.030 ± 0.012 (6/200)	0.7790	0.08
c.347 – 13 C > G (intron 4)	0.0366 ± 0.015 (6/164)	0	0.0207	5.35
c.462 + 35 G > A (intron 6)	0.1220 ± 0.026 (20/164)	0.050 ± 0.015 (10/200)	0.0219	5.25
c.648 + 21 C > A (intron 8)	0.0244 ± 0.012 (4/164)	0	0.0862	2.94
c.972 G > C (exon 12)	0.2744 ± 0.035 (45/164)	0.205 ± 0.029 (41/200)	0.1537	2.04
c.1145 – 27 C > T (intron 12)	0.0183 ± 0.010 (/164)	0.010 ± 0.007 (2/200)	0.8229	0.05
c.1435 – 40 G > C (intron 14)	0.1098 ± 0.024 (18/164)	0.060 ± 0.017 (12/200)	0.1270	2.33

MYH variants detected in our study is predicted to affect splicing. It is missense mutation p.G382D with a predicted strong 3′ splice site score.

We have found exonic splicing enhancers and intronic splice site sequences by ESEfinder 3.0. ESEfinder 3.0 indicated c.1234 C variant and c.1145 G variant as part of the exonic splicing enhancers SRp40/SRp55, and SC35, respectively. Both exonic splicing enhancers were not recognised in case of testing c.1234 T variant and c.1145 A variant.

ESEfinder 3.0 did not indicate any other significant impact on exonic splicing enhancers and intronic splice site sequences.

4. Discussion

Altogether, two *APC* mutation negative AFAP patients carried pathogenic biallelic *MYH* mutation (two of 82 probands, 2.4%) in our study. Although our group of patients isn't too big, it seems that the frequency of biallelic *MYH* mutation in our study is lower than in previous studies. The biallelic *MYH* germline mutation carriers were identified at intervals 4–36% in previous studies.[7,14] Thus, mutations in *MYH* explained only a minor part of the mutation *APC* mutation-negative polyposis cases. The remainder of the cases may be explained by mutations undetectable by the screening method, by mutations outside the coding regions of this gene and/or by mutations in other predisposing genes.

Monoallelic *MYH* variant in the coding region was found in one of the 82 patients. The frequency of monoallelic mutation carriers, however, was significantly lower in our study (0.82% compared with that of 3.9% as reported by Sieber[7]), which may reflect ethnic and geographic differences between studied populations. The risk of colorectal cancer in monoallelic *MYH* mutation carriers is still debated.[15–18] It is conceivable that germline monoallelic *MYH* mutation combined with germline mutation in other genes more strongly increase cancer risk.[19]

We have confirmed strong linkage disequilibrium between the c.462 + 35 A variant in intron 6 and the c.1435 – 40 C variant in intron 14 as was reported by Aretz and colleagues.[14] In addition, we have found strong linkage disequilibrium between the c.157 + 30 G variant in intron 2 and the c.972 C variant in exon 12 which implies a low recombination throughout the *MYH* gene.

The two variants c.347 – 13 C > G and c.462 + 35 G > A have statistically higher frequencies in the cases compared to the controls. Both variants were presented only in heterozygote status, except at one c.462 + 35 A/A homozygote in the cases.

If we excluded individuals with c.347 – 13 C > G in combination with other sequence variant, the difference in allele frequencies between cases and controls would not be significant. Accordingly, if we excluded individuals with c.462 + 35 G > A in combination with other sequence variant (except the c.1435 – 40 G > C, which is in linkage disequilibrium), the difference in allele frequencies in cases comparing to controls would not be significant as well. Hence it follows that the difference in allele frequencies between cases and controls is caused by compound heterozygotes, which is consistent with the recessive inheritance of the trait.

The identification of disease predisposing mutations is important for cancer prevention. Genetic testing for *MYH* mutation has been recommended to all patients who have tens to hundreds of colorectal adenomas with no identified germline mutation in the *APC* gene and with a family history compatible with an autosomal recessive mode of inheritance. In previous studies, *MYH* screening substantially increases the mutation detection in *APC* mutation-negative patients with adenomatous polyposis. It seems that *MYH* screening does not increase the mutation detection in unrelated Czech *APC* mutation-negative polyposis patients. Reason for lower detection of *MYH* mutation in Czech polyposis patients is unknown.

Conflict of interest statement

None declared.

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