The spectrum of the APC pathogenic mutations in Slovak FAP patients

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Abstract

OBJECTIVES: The *adenomatous polyposis coli* (APC) gene was analyzed for germline mutations in 113 familial adenomatous polyposis suspected families from all over Slovakia. Mutation screening was performed using single strand conformation polymorphism (SSCP) and DNA sequencing.

RESULTS: Mutations in the APC gene were found in 39 (34.5%) Slovak families and 25 different pathogenic mutations throughout the APC gene were identified. Of these, 12 mutations were deletion, one was insertion and 12 were base substitution.

CONCLUSIONS: Molecular diagnostics of Slovak FAP families revealed broad palette of mutations in crucial *APC* gene. The patients with identified APC gene mutations were assigned to a specific therapeutic FAP program.

INTRODUCTION

Familial adenomatous polyposis (FAP) (OMIM 175100) is one of the most clearly defined and best understood inherited type of colon cancer. The disease is characterized by the development of hundreds to thousands of colorectal adenomatous polyps and a variable range of extra-colonic manifestations. FAP is an autosomal dominantly inherited disorder predisposing to colorectal cancer (CRC) and accounts for 5% of all CRC cases. The reported incidence varies from 1 in 7 000 to 1 in 22 000 live births, with the condition being more common in Western countries (Campbell *et al.*, 1994).

FAP is caused by mutations in the adenomatous polyposis coli (*APC*; chromosome 5q21) tumor suppressor gene (Groden *et al.*, 1991). Mutations span through all *APC* exons, but a great number of all germline mutations are clustered in a relatively small region called MCR (Mutation Cluster Region) between codons 1286–1513 in the 5´prime of the largest exon 15 (Beroud & Soussi 1996). Over 95% of mutations in this region are either nonsense or frameshift mutations, resulting in a truncated protein product with abnormal function. Truncation in MCR appears to be a very important factor in adenoma development, thus molecular diagnosis of *APC* mutations usually begins in this region. The codons 1061 and

1309 are the most common germline mutation sites, and mutations at either site often result in a frameshift (Fearnhead *et al*., 2001). Codon 1309 mutations are associated with severe polyposis. Fatal outcome from colon cancer in patients bearing this mutation occurs by ten years earlier on the average compared to patients with other mutations (Caspari *et al*., 1994).

The risk of developing specific manifestations of FAP is often correlated with the position of the inherited *APC* mutation. Attenuated polyposis is usually attributed to mutations at the 5´end spanning exons 4 and 5, within exon 9 and at the 3´distal end of the gene (Soravia *et al*., 1998). Mutations restricted to 5´ of codon 169, exon 9, and 3´ of codon 1403 in exon 15 have been associated with an increased likelihood of a mild colonic phenotype (Lynch *et al*., 1993; Scott, 1995). Severe polyposis is usually seen in patients with mutations between codons 1250 and 1464 (Nagase *et al*., 1992). Mutations in codon 1309 and immediately 3´ of it tend to cause a particularly severe phenotype with earlier onset of disease (Nugent *et al*., 1994). Mutations between codons 1445 and 1578 (Caspari *et al*., 1995) or between codons 1395 and 1493 (Wallis *et al*., 1999) have been associated with extracolonic manifestations (desmoids, osteomas, epidermoid cysts and upper gastrointestinal polyps).

The most frequently used diagnostic technique for screening of *APC* gene mutation is single strand conformation polymorphism (SSCP). SSCP is a suitable method for detection not only of 2–5 bp deletions but of 1 bp deletion, insertion and substitution as well (Orita *et al*., 1989). In our analysis of Slovak FAP patients, we used SSCP and direct sequencing to identify germline mutations in the *APC* gene. Special abnormalities detected by SSCP were analyzed by WAVE System (Transgenomic Biosystem, USA).

PATIENTS AND METHODS

Patients

Members of 113 families suspected of adenomatous polyposis were examined for mutations in the *APC* gene. The families were collected in collaboration with the National Cancer Institute in Bratislava and several local hospitals all over Slovakia. Written informed consent for genetic testing was obtained.

DNA isolation and PCR amplification

Genomic DNA was prepared from peripheral blood lymphocytes using QIAamp® DNA blood Kit (Qiagen). DNA samples were amplified using PCR mostly in the program: 5 min at 94 °C, once; 1 min at 94 °C, 1 min at annealing temperature – 58 to 63 °C, and 1 min at 72 °C, 30 times; and 7 min at 72 °C, once.

Single strand conformation polymorphism.

PCRs for SSCP were performed from approximately 150–200 ng of genomic DNA, 80 mM dNTP, 1 mM 10 × PCR buffer (Qiagen), 0.5 U of Taq polymerase (Qiagen), 10 pmol of each primer, to a total volume of PCR mixture of 25 µl. The sequences of the primers used for the *APC* gene were described by Groden *et al*., (1991). The samples were denaturated for 5 min. at 95 °C, then placed on ice for 5 min. in order to prevent reannealing, loaded onto a 6% polyacrylamide gel and electrophoresed at 10 °C at 55 V overnight. The gel was silver-stained as described (Kirchhoff *et al*., 1997): 15 min. fixation in 10% ethanol, 10 min. incubation with 1% HNO_3, 30 min. incubation with 0.2% $AgNO_3$ containing 1 µl/ml formaldehyde, developed with 3.5% Na_2NO_3 containing 0.5 µl/ml formaldehyde until the bands appear, and fixation with 10% acetic acid. The gel was then dried.

DHPLC (WAVE System)

Denaturing high-performance liquid chromatography was carried out on automated HPLC instrumentation equipped with a DNA Sep column (Transgenomic Inc., San Jose, CA). WAVE System is one of the most reliable and used DNA mutation detection system with nearly 100% effectivity.

Direct DNA sequencing.

Amplicons were purified by solid-phase extraction and bidirectionally sequenced with the PE Applied Biosystems Big Dye Terminator Sequencing Kit according to the manufacturer's instructions. Sequencing extensive products were analyzed on a PE Applied Biosystems ABI-PRISM 310 sequencer (Zajac *et al*., 2007).

RESULTS

In this study SSCP and sequencing were used to investigate the *APC* gene for germline mutations in 113 families suspected of familial adenomatous polyposis. Mutations were found in 39 families (34.5%). Summary of DNA alteration identified in these patients is given in Table 1.

The 24 types of mutations were spread across the *APC* gene with the majority clustered in exon 15. A great number (52%) of these mutations were frameshift mutations caused by deletion or insertion of a small number (2–5) of nucleotides. All mutations were out of frame and therefore resulted in downstream premature chain terminating signal. Only one of this frameshift mutations was caused by large deletion of 29bp, beginning at nucleotide position 759 through to 787 (family no. 141). This mutation is in exon 7 and is associated with a classic form of FAP with late onset of the disease. The common mutation at codon 1309 was identified in six families and at codon 1061 in three families.

A rather unusual mutational event was identified in family 154. Sequencing analysis revealed 2 bp deletion and 1 bp insertion (638_639delAGinsT). The result of these mutational events was the production of a stop signal (TGA) in codon 218. Such unusual combination of two mutations is not registered in the mutation database.

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Forty–eight percent of all detected mutations were single base substitutions. The most common single base changes were a C to T transition, converting an arginine amino acid to a stop codon (TGA) and transversion C to G, converting a serine amino acid to the stop codon (TGA). Missense mutation was found in ten families, converting aspartic acid to valine amino acid in codon 1822. In two families, along with this missense mutation also a nonsense mutation was found. In family 157 this nonsense mutation in codon 1096 was causing C to T transition, converting amino acid glycine to a stop codon (TAG) and we did not find any references concerning this mutation in the mutation database. In family 109 was found nonsense mutation in codon 216 and was causing amino acid arginine to a stop codon conversion. All single base changes were revealed by the SSCP and/or WAVE System.

DISCUSSION

Since colorectal cancer is easily treatable if it is found in the premalignant stage of adenomas, participation in regular colon screening is important for individuals at high risk. Cancer risk assessment and genetic testing are the most effective ways of determining individual and family risk.

Single strand conformation polymorphism, with a sensitivity of approximately 80%, was used as the basic screening technique. Some *APC* mutations not exactly determined by SSCP were re-analyzed by WAVE Mutation Detection System. Genetic testing for FAP can be performed on individuals with polyposis, as well as on at-risk individuals, provided a mutation in an affected person has been identified. The practice of testing children for a germline *APC* mutation is acceptable because

Table 1. Summary of *APC* sequence alterations identified in Slovak FAP patients

Exon	Codon	Nature	Alteration	Consequence	Family No
2	Splice site	Base substitution	t to c	Frameshift	152
5	213	Deletion/Insertion	delAGinsT	Frameshift (TGA)	154
5	213	Base substitution	C to T	Arg to stop (TGA)	104
6	216	Base substitution	C to T	Arg to stop (TGA)	109
7	253	Deletion	29bp	Frameshift (11, TGA)	141
8	283	Base substitution	C to T	Arg to stop (TGA)	9
8	291	Deletion	TT	Frameshift (3, TAG)	151
15B	753	Deletion	C	Frameshift (7, TAG)	139
15B	788	Deletion	A	Frameshift (31, TGA)	136
15B	811	Deletion	ACAA	Frameshift (6, TGA)	106
15B	837	Base substitution	C to G	Ser to stop (TGA)	59
15C	933	Deletion	ACTT	Frameshift (20, TAG)	128
15E	1061	Deletion	ACAAA	Frameshift (2, TGA)	29, 41, 96
15E	1096	Base substitution	C to T	Gly to stop (TAG)	157
15E	1102	Deletion	ACAG	Frameshift (22, TAA)	11
15E	1138	Deletion	A	Frameshift (26, TAA)	47
15F	1209	Base substitution	G to T	Glu to stop (TAA)	2
15F	1249	Base substitution	C to A	Cys to stop (TGA)	3
15F	1272	Base substitution	C to G	Ser to stop (TGA)	16
15G	1309	Deletion	AAAGA	Frameshift (4, TAG)	10, 27, 38, 91, 93, 132
15G	1344	Base substitution	C to G	Ser to stop (TGA)	70
15H	1444	Deletion	T	Frameshift (29, TAA)	65
15H	1450	Base substitution	C to T	Arg to stop (TGA)	156
15I	1554	Insertion	A	Frameshift (4, TGA)	116
15L	1822	Base substitution	A to T	Asp to Val	109, 115, 117, 124, 125, 127, 128, 129, 131, 157

polyps develop at a young age (Zawacki, 2002). Children with a negative result can be spared years of invasive colon screening, while children who test positive can be more vigilant about screening.

By using a combined strategy of SSCP, WAVE System and direct DNA sequencing, we found several mutations in our patients. The most frequent mutations in Slovak families with FAP were 3927–3931delAAAGA (del 5 bp at 1309), occurring in 6 (15.4%) families and 3183–3187delACAAA (del 5 bp at 1061), which was seen in 3 (7.7%) families (Zajac *et al*., 2002). Codons 1061 and 1309 are mutational hot spots and account for approximately 11% and 17% of all germline mutations (Sieber *et al*., 2000). Somatic mutational hot spots occur at codons 1309, 1450 and 1554, accounting for approximately 7%, 8% and 5% of all somatic mutations respectively (Sieber *et al*., 2000). In family 156 we found germline mutation in one of these somatic mutational hot spots, in codon 1450. This mutation is associated with a profuse type of polyposis (>5000 colorectal polyps) and with extra-colonic manifestations.

Base substitution (C to T), which we found in codon 1450, is very frequent in the European population. This transition mainly occurs at CGA codons and leads to the nonsense mutation TGA. We found this mutation in three families (9, 104, 109), differing in their phenotype. In family 104 the mutation was localized in codon 213 and was associated with an attenuated form, while in family 9 (mutation in codon 283) and 109 (mutation in codon 216) the mutation was associated with a classic form of FAP.

Mutation in the region between codons 1445 and 1578 (Caspari *et al*., 1995) or between codons 1395 and 1493 (Wallis *et al*., 1999) are associated with the occurrence of numerous features outside the colon (desmoid tumors, osteomas, epidermoid cysts, and upper gastrointestinal polyps), which are classified as Gardner syndrome. We identified three mutations in these regions: 4331delT in family 65, 4660insA in family 116 and 4348subC>T in family 156. Two of these families (65, 116) had features of Gardner syndrome, while about family 156 no data were available.

Missense mutation in codon 1822 (5465subA>T) is very frequent in Slovak families. The significance, if any, of this polymorphism is unknown. However, as it is located in the middle of the β-catenin down regulated domain, it may result in disruption of the putative cell signaling function of the APC protein (Munemitsu *et al*., 1995). Mutation in codon 1822 (pD1822V) is a missense variant previously shown to be a common polymorphism unrelated to the risk of developing CRC or colorectal adenoma (Ruiz-Ponte *et al*., 2001; Tranah *et al*., 2005). However, two previous studies of CRC demonstrated significant gene-environment interactions between the pD1822V polymorphism and consumption of a low-fat diet and postmenopausal hormone use (Tranah *et al*., 2005; Slattery *et al*., 2001). It seems that AT heterozygotes for this site had a low odds ratio for CRC. We suppose that families with this polymorphism are at-risk individuals and it is important for them to be integrated into a screening and therapeutic program. In families 117, 129 and 131 we analyzed the whole *APC* gene by direct sequencing and found only this missense mutation. Members of these families have the classic form of FAP. On the other hand, in two families (109, 157) we found besides this mutation a nonsense mutation too, which resulted in a truncated nonfunctional protein. We concluded that the nonsense mutation was a causative genetic factor of FAP.

In our study we found mutations in 34.5% of the families studied. The lower percentage of detected mutations could have been caused by the occurrence of mutations outside the studied region or the lower efficiency of the methods used to detect mutations. In families in which we did not find germline mutation in *APC* gene we also screened *p53* and *CTNNB1* genes for mutation (Kovac *et al*., 2005). No mutations in these genes were found.

Possible mechanism of induction of the *APC* mutations is very intensively studied. We plan to observe the role of intestinal bacteria in this process (Zajac *et al*., 2006).

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