Analysis of bacteria from intestinal tract of FAP patients for the presence of APC-like sequences

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Summary

Background: Familial adenomatous polyposis (FAP) is a hereditary disease induced by germ-line mutations in the tumor suppressor APC gene. These initiate the early stages of the adenoma-carcinoma sequence in familial, but also in sporadic (in 80% to 90%), colon tumorigenesis. We found the presence of APC-like sequences in bacteria of FAP patients.

Material/Methods: We analyzed bacteria isolated from FAP patients’ rectal swabs. Total bacterial DNA was isolated and analyzed for detection of APC-like sequences using PCR. We also tested DNA homology rate and APC-like protein production.

Results: We collected blood samples and rectal swabs from patients with confirmed diagnosis of FAP. They were analyzed for presence of sections from exon 15 of the APC gene. Most positive results were found in sections located exactly in the area called the MCR (mutation cluster region), where the highest frequency of APC gene mutations were identified. By sequencing PCR products from bacteria in section F-G together with a patient’s DNA sample and human APC gene, we found a more than 90% DNA homology rate. We also confirmed production of APC-like protein using Western blotting.

Conclusions: Our results suggested two hypotheses. The APC-like protein might have same function as a truncated APC product, which is synthesized in most cases of mutations of APC gene in the MCR region in colorectal cancer cells. Alternatively, we can consider the possible existence of horizontal transfer of genetic information between eukaryotic and prokaryotic cells. Our study can be considered as a pilot project. For confirmation of our hypotheses, further research is needed.

key words: APC-like sequences • familial adenomatous polyposis • colorectal cancer • bacteria • mutation cluster region

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**Background**

Cancers of the digestive tract are among the foremost causes of mortality, not only in Slovakia, but also worldwide [1]. Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by development of hundreds or thousands of polyps in the gut, with an almost 100% probability of degeneration into malignant processes in the second decade of life. This disease is induced by germ-line mutations in the tumor-suppressor APC (adenomatous polyposis coli) gene [2], which normally inhibits cell growth. An inactivating mutation of this gene is also present in up to 75–80% of sporadic colorectal adenomas and carcinomas [3,4]. The spectrum of these somatic mutations is very similar to that of the germ-line mutations [4]. The most common disease-causing mutations (95% of all known) create stop codons or frame shifts [5], which result in the lost of APC protein function, which is a critical event in the process of carcinogenesis. The APC coding region consists of 8535 bp and is divided into 15 exons. The majority of the approximately 700 detected pathogenic APC mutations [6] occur in exon 15 and over 60% of these are in the region between codons 1286–1513 [7], also known as the Mutation Cluster Region (MCR), which accounts for less than 10% of the coding region [8].

The high concentration of APC gene mutations in region 220 bp raises the question of their genesis. This phenomenon is frequently explained by the weakening of the region due to its specific primary DNA structure. There are 2 critical spots in the MCR region (2 specific hypopolymers sites): 1) sequences AAAAGAAAGA (codons 1307–1311), where deletion of AAGAA or AAGGA is very frequent; and 2) the second hotspot mutation is a 1-bp deletion that occurs within the repeat sequence CCTAAAAATAA (bp 4378–4382). These 2 mutations are very probably induced by a polymerase slippage error within repeated nucleotide sites [9]. Another explanation is based on functional definition. Each mutation in the MCR leads to the synthesis of a truncated APC product, which is able to provide some residual function despite the lack of any β-catenin degradation activity. But for keeping this function it must contain at least the first 20 amino acid repeat domain, so the 5’ border of the MCR is located right after this area imposed by the necessity of controlling the activity of β-catenin in a cell cycle-dependent manner [10].

Factors that lead to mutations and tumorigenesis of colorectal cancer are not strictly defined yet, so the need to analyze them is still very real. The idea that bacteria may play an important role in the formation and development of colorectal cancer is currently widely accepted [11–14]. Moore reported that 15 bacterial species were significantly associated with a high risk of colon cancer [15] and other authors have recently published reports on which microorganisms cause cancer in humans [16–18]. Based on detection of APC-like sequences in intestinal bacteria isolated from FAP patients, we present a new hypothesis/approach regarding the induction of the APC gene mutation and/or colorectal tumorigenesis. Presence of foreign DNA sequences (HIV) in bacteria have been previously reported [19].

**Material and Methods**

**DNA extraction**

Genomic DNA was extracted from peripheral blood lymphocytes of FAP patients using the QIAamp DNA blood Kit (Qiagen). Bacteria isolated from rectal swabs were amplified overnight in LB medium and bacterial clones were prepared after dilution on LB agar plates. Bacterial chromosomal DNA was extracted by use of the QIAamp DNA Kit (Qiagen). Plasmid DNA was isolated with the manual QIAprep Spin Miniprep Kit (Qiagen).

**Primers and PCR**

The sequences of the primers used for the APC gene were described by Groden et al. [2]. PCRs 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. DNA samples were amplified using the cycling program: 5 min at 94°C, once; 1 min at 94°C; 1 min at annealing temperature from 58° to 63°C; 1 min at 72°C, 30 times; and 7 min at 72°C, once.

**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.

**Analysis of the APC gene expression**

Bacterial protein extracts of tested FAP patients were prepared from 15 ml overnight cultures, that were transferred into 25 ml LB medium with kanamycin and let cultures grow to optimal optical density. Cultures were divided into two parts – one with IPTG to final concentration of 1 mM IPTG/ml and one without IPTG. Both cultures were grown overnight at 37°C and protein extracts were prepared by centrifugation at 3000 rpm for 15 min. Pellets were sonicated and 10 µl of 100 mM PMSF was added and centrifuged at 15000 rpm for 15 min. Prepared cell extracts were electrophoresed in 10% SDS-PAGE. Bacterial protein extracts for positive control were prepared similarly from 3 ml of overnight cultures of clone DE3pLys bearing plasmid with cloned complete APC gene (paper prepared for publication).

**Western blotting**

Proteins after electrophoresis were transferred from acrylamide gel to nitrocellulose (NC Hybon membrane) overnight at 50 mA. Membrane was incubated in TBS-T buffer and blocked using 5% milk for 1 hour at room temperature. The blocking buffer was removed and the membrane was washed with TBS-T buffer. Appropriately diluted mouse monoclonal APC antibody (ALi 12–28, Abcam) and rabbit polyclonal antibody to APC (ab15270) in TBS-T buffer with 5% milk was added to the membrane and incubated overnight at 4°C on a shaker. The membrane was washed with TBS-T buffer and incubated for 1 hour at room temperature in appropriately diluted goat
anti-mouse antibody (sc-2005), or goat anti-rabbit IgG-HRP: sc-2030 (Santa Cruz Biotech.) in buffer containing 5% milk respectively. Consequently, the membrane was washed with TBS-T buffer and ECL solutions were used for visualization.

**RESULTS**

We collected blood samples and rectal swabs from 15 patients with a confirmed FAP diagnosis and from 4 healthy persons. Bacteria isolated from rectal swabs were amplified overnight in LB medium and each subclone was analyzed for the presence of APC-like sequences using PCR. We analyzed sections A, D, E, F, G, H, H–J, K–N a Q from exon 15 of the APC gene. As a positive control we used a human DNA sample from a healthy person. Laboratory strain HB 101 and bacteria isolated from the gastrointestinal tract of healthy persons (100-5, 77-1, K17-2-5, 164-1) were used as negative controls in all reactions.

PCR products from APC gene defined by primers 15Afor-15Arev were identified in bacteria isolated from patient 38-3 and in bacterial subclone 41-1-23 from patient 41-1 (Figure 1, lines numbers 8, 9). Similar results were observed by analysis of section defined by primers 15Dfor-15Drev, but PCR product were also found in samples 60-3 and 104-1 (Figure 2, lines numbers 8, 9). By analysis of the sections 15Efor-15Erev and 15Ffor-15Frev, PCR products were identified by all samples except for patient 46-2 in both sections and 96-3 in section 15Efor-15Erev (Figure 3, lines 12). In section 15Gfor-15Grev, positive results were by all tested samples (Figure 4). Results in section 15Hfor-15Hrev were positive by samples 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174\(\text{HaeIII}\).

**Figure 1.** PCR analysis of bacterial DNA isolated from FAP patients determined by primers 15Afor-15Arev. Lines 1–18: 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174\(\text{HaeIII}\).

**Figure 2.** PCR analysis of bacterial DNA isolated from FAP patients determined by primers 15Dfor-15Drev. Lines 1–18: 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174\(\text{HaeIII}\).

**Figure 3.** PCR analysis of bacterial DNA isolated from FAP patients determined by primers 15Efor-15Erev. Lines 1–18: 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174\(\text{HaeIII}\).

**Figure 4.** PCR analysis of bacterial DNA isolated from FAP patients determined by primers 15Gfor-15Grev. Lines 1–18: 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174\(\text{HaeIII}\).
positive results were recorded for samples 38-1, 38-1-7, 41-1-37, 46-2, 55-1, 55-2, 55-3, 56-2, 96-3, 155-1 and 104-1. By analysis of the last two sections results were almost the same and positivity in section 15K for 15N rev was recorded only for samples 55-2 and 104-1 and in 15Q for 15Q rev only one positive result was observed in sample 55-2. All results are clearly presented in Table 1.

PCR products from bacteria, respectively from their subclones, from patient 41-1 were sequenced in section F-G together with the patient’s DNA sample and the sequence of human APC gene and subsequently compared using the computer program. The result was more than 95% of the rate of homology in this section (Figure 6).

In the last part of our project we used seven samples (55-1, 55-2, 55-3, 60-3, 96-3, 104-1, 41-1) for determining the possible expression of APC-like sequences, respectively possible production of APC-like protein. We used Western blotting with monoclonal and polyclonal antibodies for APC.

Table 1. Distribution of PCR products positivity synthesized on the template of bacteria isolated from rectal swabs of FAP families members.

| Patient’s samples | A–A | D–D | E–E | F–F | G–G | H–H | H–J | K–N | Q–Q |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 38-1            |     |     | +   | +   | +   | +   | +   |     |     |
| 38-1-4          |     |     | +   | +   | +   | +   | +   |     |     |
| 38-1-7          |     |     | +   | +   | +   | +   | +   |     |     |
| 38-1-16         |     |     | +   | +   | +   | +   |     |     |     |
| 38-2            |     |     | +   | +   | +   |     |     |     |     |
| 38-3            |     | +   | +   | +   | +   |     |     |     |     |
| 41-1            |     | +   | +   | +   | +   |     |     |     |     |
| 41-1-19         |     | +   | +   | +   | +   |     |     |     |     |
| 41-1-23         |     | +   | +   | +   | +   |     |     |     |     |
| 41-1-26         |     |     | +   | +   | +   |     |     |     |     |
| 41-1-32         |     |     | +   | +   | +   |     |     |     |     |
| 41-1-33         |     |     | +   |     |     |     |     |     |     |
| 41-1-37         |     |     | +   |     |     |     |     |     |     |
| 46-2            |     |     |     |     | +   | +   |     |     |     |
| 46-4            |     |     |     |     | +   | +   | +   |     |     |
| 55-1            |     |     |     | +   | +   | +   |     |     |     |
| 55-2            |     |     |     |     | +   | +   | +   |     |     |
| 55-3            |     |     |     |     |     | +   | +   | +   |     |
| 56-1            |     |     |     |     | +   | +   | +   |     |     |
| 56-2            |     |     |     |     |     | +   | +   | +   |     |
| 60-3            |     |     |     | +   | +   | +   |     |     |     |
| 96-3            |     |     |     |     |     |     |     |     |     |
| 155-1           |     |     |     |     |     |     |     |     |     |
| 104-1           |     |     |     |     |     |     |     |     |     |

Figure 5. PCR analysis of bacterial DNA isolated from FAP patients limited by primers 15Hfor-15Hrev. Lines 1–18: 38-1, 38-1-4, 38-1-7, 38-1-9, 38-1-12, 38-1-16, 38-2, 38-3, 41-1-23, 41-1-26, 41-1-37, 46-2, 46-4, 55-1, 55-2, 55-3, 56-1, 56-2; 19: positive control human DNA of patient 100-5; 20: negative control HB 101; 21–24: healthy persons 100-5, 77-1, K17-2-5; 25: marker øX174 HaeIII.
Gene. Protein products were observed in samples 55-3, 60-3, 96-3, 104-1, 41-1 (Figure 7).

**Discussion**

Bacteria isolated from rectal swabs of FAP patients and their subclones were analyzed for the presence of APC-like sequences using PCR. We analyzed exons 9 to 14 without PCR products detection. The PCR products were found in exon 15, in which were analyzed sections A, D, E, F, G, H, H–J, K–N and Q. In bacteria or their subclones isolated from 15 members of 9 FAP families were observed APC-like sequences mostly in sections E, F, G and H. This is very interesting, because this part of the APC gene corresponds with MCR. Moreover, sequencing and subsequent software comparison showed about 90% of homology with APC gene. For possible assignment of expression of these sequences, monoclonal and polyclonal antibodies were used, which visualized protein at about 40 kDa in some samples.

About 90% of tested bacteria were identified as *E. coli*. Some clinical studies have explored the association between this bacterial strain and colorectal cancer. It was proven that both extracellular and intracellular bacterial counts of *E. coli* were increased in patients with colonic adenoma or carcinoma [20]. In another study, increased adherence and invasion of *E. coli* was reported in patients with colorectal cancer and Crohn’s disease [21]. These results might be considered direct evidence of their intracellular invasion in these patients.

The presence of APC-like sequences in commensal bacteria of the patients may be hypothetically explained as follows: 1) genetic information including APC-like sequences was accepted by bacteria in the intestinal tract from degraded human cells, in particular by macrophages and lymphocytes. Acceptance of new genetic information is a basic feature of bacteria, because richer genetic material gives them a better chance to survive. 2) APC-like sequences are an integral part of intestinal bacteria. They may represent a very conservative part of the genome; this original primary structure of the APC gene – the first 14 small exons and the large 15 exon – might also serve as a “matrix” for formation of tumor suppressor genes in the highest organisms in the evolutionary process.

Possibly a function of APC-like sequences, APC-like protein might also be explained in other ways. APC is a multifunctional protein [22] involved in cell adhesion [23], polarity, migration, mitosis, apoptosis, and neuronal differentiation and plays a key role in so-called β-catenin destruction complex, catalyzing the efficient phosphorylation of β-catenin, which is subsequently degraded in the proteasome [24]. Therefore inactivation of its physiological function in colon cancer cells leads to the stabilization of β-catenin, which in turn gives the cell a permanent mitogenic signal [25, 26]. This is the essential mechanism allowing the hyperproliferation of the epithelial cells of the colon [27–29].

The mutation cluster region (MCR) of the APC gene is located within the central part of the open reading frame, including the so-called 20 amino acid repeats (20R) that are β-catenin binding sites. Each mutation in this area leads to the synthesis of a truncated APC product expressed in a colorectal tumor. In FAP, one mutated allele is germ-line, but the sequence of mutational events affecting both alleles is in this case not a random process [30–32] and we can observe strong selection for the retention of at least one truncated APC product containing the first 20R (germ-line defect – loss of heterozygosity or a truncating mutation occurring
before the first 20R, then the next truncating mutation will affect the remaining allele after the first 20R). Therefore the 5’ border of the MCR is clearly located just after the first 20R; we have recently shown that it is an important structural motif allowing truncated APC to keep some residual control of β-catenin activity [33] for optimal cell proliferation [34]. The 3’ border of the MCR is less well defined. But there is also an almost systematic selection for truncating mutations occurring before the SAMP, probably to preclude the formation of a functional destruction complex by eliminating the binding sites for axin/conductin [35], which involve β-catenin degradation. Or the selection might depend on altered β-catenin binding activity to the third 20R, which contains 2 parts that provide important contact sites to β-catenin [36,37]. Therefore almost all APC gene mutations that are selected in colorectal tumors lead to the synthesis of truncated APC products that contain a 20R1 fully competent in β-catenin binding. These facts shows, that some β-catenin binding activity must be kept and that it is crucial for the tumor cell [10] and the presence of truncated APC seems essential for optimal cell proliferation [34], but all the consequences in terms of intracellular signalling remain still unknown.

On the basis of all these facts we can consider the role of APC-like sequences, respectively APC-like proteins produced by bacteria as an alternative origin of truncated APC products. Almost all our samples were positive for the APC-like sequences in the sections E-E and F-E, which are the particular regions where the 20R1 is encoded. If APC-like protein contains this section, respectively β-catenin binding side, then it is probably able to bind to β-catenin and this controls its ability to activate transcription, just like truncated APC products. Moreover, because of this interaction, the binding domains are occupied by these molecules and are not able to bind with functional APC protein to provide its physiological function. Thus, APC-like proteins could probably also operate by the mechanism of competitive inhibition, which might explain the tumor formation in situations in which no mutation of APC gene is detected.

Extracellular DNA has already been found in untreated cultures of a variety of micro-organisms [38,39] and in the culture medium of mammalian cells [40–42]. Furthermore, extracellular DNA that has been spontaneously released by bacterial species amenable to genetic transformation has been shown to be genetically active [39,43]. Anker et al reported that when human cells and bacteria are both present together, as can be the case during septicemia, peritonitis, or subclinical inflammation of the gut, human cells can take up bacterial DNA [44]. Both cellular [45] and nuclear membranes [46] can be crossed by nuclear material and this enables it to gain access to the host genome [47]. Thus, bacterial DNA transcribing into human cells might have medical implications, especially in the process of carcinogenesis [44]. It is possible that the role of bacteria in oncogenesis, such as demonstrated with helicobacter and gastric MALT lymphoma or gastric carcinoma [48,49], is underestimated. Accepted APC-like sequences are not vital for bacteria, so their mutations are without implications for them. But, hypothetically, bacteria with mutated APC sequences might be internalized by human cells, including sperm and oocytes, and mutation could be horizontally transferred by in vivo DNA hybridization, and consequently integrated into the human genome where it may create somatic or germ-line mutations.

**Conclusions**

We do not know yet, if confirmation of the presence and function of APC-like sequences in bacteria could be beneficial in analyzing the causes of the disproportionate number of mutations in the APC gene. Our results are quite preliminary and they can be considered only as hypothesis-generating because of the limited number of analyzed samples. Our study is a pilot project and further research is warranted. If confirmed, this original model could bring new opportunities in research, diagnostics and even therapy of this disease.

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