Influence of diet containing lyophilized *Enterococcus faecium* M-74 with organic selenium on tumor incidence in *Apc*^{1638N} mice*

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The aim of the present study was to test the effect of long-term application of diet containing *Enterococcus faecium* M-74 with organic selenium on tumor induction in transgenic mice carrying mutation in *Apc* gene. Heterozygosity for the *Apc*^{1638N} mutation in mice causes development of small intestine and gastric tumors. Feeding of *Apc*^{1638N} transgenic mice with enriched diet with probiotic components during 8 months have shown a minor therapeutic effect on the clinical manifestations in small intestine in comparison with control group.

Key words: Apc gene mutation, transgenic mice, probiotic diet

Mutation of the tumor suppressor adenomatous polyposis gene (*Apc*) represents a very early event in the multi-step process of colorectal cancer progression. Germ-line mutations in *Apc* gene are responsible for predisposition to familial adenomatous polyposis (FAP). FAP has an incidence of about 1 in 7000 [8]. Individuals who inherit a single copy of a mutant *Apc* gene develop hundreds to thousands of colonic polyps. These lesions may progress to adenomas and carcinomas. FAP patients also exhibit number of other symptoms, for example congenital hypertrophy of the retinal pigment epitelium (CHRPE), desmoid tumors of the skin, medulloblastomas and osteomas [11, 19]. Somatic mutations in *Apc* gene have also been observed in more than 80 percent of sporadic adenomas and carcinomas [16].

FODDE et al [5] generated a unique *in vivo* model, *Apc*^{1638N} transgenic mice. A neomycin resistance cassette was inserted into exon 15 at a site corresponding to codon 1638 of the mouse *Apc* tumor suppressor gene. This mutation results in an unstable, truncated 182 kD protein [9]. While homozygosity *Apc*^{1638N}/*Apc*^{1638N} is embryonic lethal, inbred B6 mice that are heterozygous *Apc*$^{+}$/*Apc*^{1638N} develop 5–6 intestinal adenomas and adenocarcinomas predominantly in the upper intestinal tract, within the first 6 months of life [1, 6, 24]. In addition to the intestinal tumors, *Apc*^{1638N} model is characterized by a broad spectrum of extraintestinal manifestations including multifocal desmoids, cutaneous cysts [18, 20, 21] and abnormalities of the retinal pigment epithelium [12]. Somatic mutation analysis of the intestinal *Apc*^{1638N} tumors has revealed loss of the wild type copy in >75% of the lesions [19].

Relationship between intestine bacterial flora and colorectal cancer has been objects of investigation over 30 years. In recent years the probiotic bacteria as a component of the intestine flora are in focus. A series of studies in animals have been performed with probiotic species that consistently show protection against the development of gastrointestinal tract cancer [4, 12, 14, 23]. The mechanisms of these effects are not clear, but could include inactivation of mutagens and carcinogens [12], enhanced immune response [7, 13, 22, 23], alteration of metabolic activities of the intestinal microflora, binding, and degradation of potential carcinogens [2, 14, 15, 16]. It appears that probiotics with or without prebiotics have an inhibitory effect on the development of precancerous lesions and tumors in animal models [2].

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In the present study we investigated the long-term effect of diet composed from lyophilized probiotic bacteria *Enterococcus faecium* M-74 and organic selenium on the incidence of intestinal adenomas in Apc^{+}/Apc^{1638N} transgenic mice.

Material and methods

Animals. Wild type Apc^{+}/Apc^{+} (C57BL/6) and heterozygous Apc^{+}/Apc^{1638N} transgenic mice were kindly provided by the Experimental Animal Farm of the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic with permission of Prof. Jan Svoboda. Mice were housed in a room with a 12-h light-dark cycle and had free access to food and water. All experimental procedures were approved by the Ethical Committee of the Cancer Research Institute in Bratislava.

Genotyping of mice. Genomic DNA was isolated by standard techniques from tail snips of 3 weeks old animals. All mice were typed for their *Apc* status by single PCR reaction [6]. Three oligonucleotide primers were used in PCR under standard conditions (94 °C for 5 min, 40 cycles of 94 °C for 1 min, 58 °C for 45 sec, 72 °C for 45 sec and 10 min at 72 °C).

Primer A: 5'-TGCCAGCACAGAATAGGCTG-3'
Primer B: 5'-TGGAAGGATTGGAGCTACGG-3'
Primer C: 5'-GTTGTCATCCAGGTCTGGTG-3'

Primer pairs AC detected wild type allele (transcript 300 bp) and primer pairs AB mutated Apc^{1638N} allele (transcript 400 kp) (Fig. 1).

Experimental design. Mice after genotyping were divided in four groups. Both Apc^{+}/Apc^{1638N} and Apc^{+}/Apc^{+} mice were maintained on either normal diet (Dobrá voda, Slovak Republic) or diet enriched with *Enteroccocus faecium* with selenium (15mg/kg/day containing 15 μg of organic selenium). Lyophilized *Enterococcus faecium* strain M-74 enriched with selenium was kindly provided by Dr. Petr Mičan, Medipharm, Hustopeče, Czech Republic. Enriched diet was repeatedly prepared fresh, in periods of three weeks. Lyophilized *Enteroccocus faecium* M-74 cells were mixed with the powdered food. Prepared pellets were dried at 50 °C what did not destroy the viability of bacteria as was controlled by tests at the Institute of Cell Biology, Faculty of Science, Comenius University, Bratislava, Slovak Republic. The dose of probiotic bacteria in the diet was as described previously by ROVENSKY et al [17]. The animals started to consume the diet being at average 2 months old for at least 8 months.

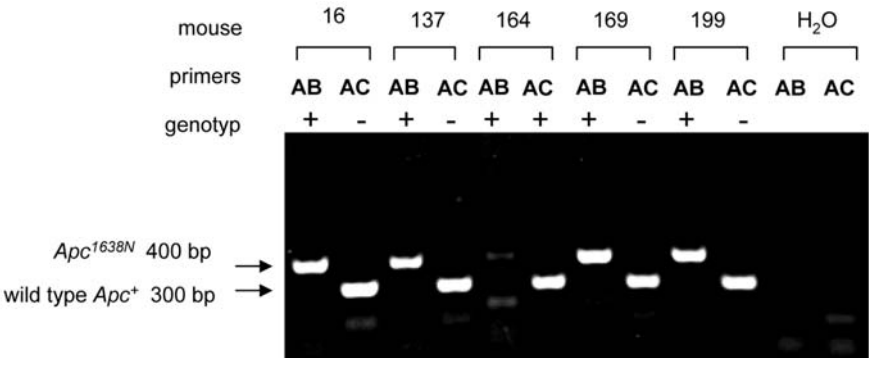

Figure 1. PCR analysis of genomic DNA derived from homozygous Apc^{+}/Apc^{+} and heterozygous Apc^{+}/Apc^{1638N} mice. Primer pairs AB detect the mutated Apc^{1638N} allele. Primer pairs AC detect the wild-type allele.

Table 1. Effect of consumption of diet enriched with *Enterococcus faecium* M-74 on viability of Apc^{+}/Apc^{1638N} mice

Experimental groups	Diet	No. of mice sex	No. of mice total	No. of mice with intestinal lesions	No. of dead mice (7 months old)
Apc+/Apc^{+}	standard	13 ♀ 5 ♂	18	0	0
Apc+/Apc^{1638N}	standard	7 ♀ 13 ♂	20	20 (100%)	7 (35%)
Apc+/Apc^{+}	M-74 enriched	12 ♀ 12 ♂	24	0	0
Apc+/Apc^{1638N}	M-74 enriched	17 ♀ 7 ♂	24	24 (100%)	5 (20%)

Histopathological analysis. Mice were euthanized and the entire gastrointestinal tract was removed. All small intestines were opened longitudinally, rinsed with physiological saline and inspected for neoplastic lesions under stereomicroscope. The number and location of lesions in duodenum, ileum and jejunum was recorded. Tissue samples were routinely processed to paraffin blocks for histological analysis.

Results

Mice kept on normal diet. There were no changes in the small intestine observed in mice with genotype Apc^{+}/Apc^{+}. All 18 mice lived to the end of the experiment (approx. 11 months). On the other hand, the group of all 20 heterozygous mice Apc^{+}/Apc^{1638N} had their small intestine attacked in all 20 cases (Tab. 1) Localization of tubular or tubulovillous adenomas was 75% in duodenum, 18% in jejunum and 7% in ileum, an average number of lesions was 3.8 per an animal (1–11). The most frequent localization, up to 85%, was in duodenum nearby the gastrointestinal junction, 7 male animals died (35%) at the age of approx. 7 months,

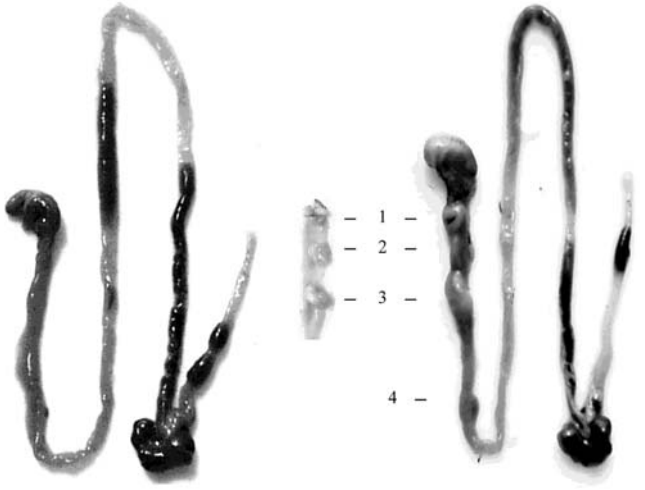

Figure 2. Gastrointestinal tracts. (A) homozygous mouse *APC*$^{+}$/*Apc*$^{+}$. (B) Four adenomas located in the duodenum of heterozygous *Apc*$^{+}$/*Apc*^{1638N} mouse.

autopsy, if possible, showed presence of large adenomas, as large as 2 cm.

Mice kept on diet enriched with Enterococcus faecium M-74 and organic selenium. Similarly as in the previous group of mice with genotype *Apc*$^{+}$/*Apc*$^{+}$, no changes in all 24 animals were observed. All animals survived the experiment and lived to the age of approx. 11 months. The group of heterozygous *Apc*$^{+}$/*Apc*^{1638N} mice had in all 24 cases attacked small intestine (Tab. 1 and Fig. 2). Localization of tubular or tubulovillous adenomas was 70% in duodenum, 17% in jejunum and 13% in ileum, an average number of lesions was 3.5 per an animal (1–6). The most frequent localization, up to 85%, was in duodenum nearby the gastrointestinal junction, 5 male animals died (20%) at the age of approximately 7 months. Autopsy demonstrated that the death was most likely caused by large adenomas in the small intestine, which caused its impassability.

Tubular and tubulovillous adenomas in the group of heterozygous *Apc*$^{+}$/*Apc*^{1638N} mice were observed regardless diet used (Fig. 3).

Discussion

Germ line mutation in tumor suppressor gene *Apc* in humans is an introductory mutation in predisposed individuals to occurrence of adenomas on colon, which then progress to adenocarcinoma due to subsequent mutations of other genes. In contrary to the transgenic mouse model carrying the mutation in *Apc* gene, the outcome is adenomas in the upper intestine. While the use of probiotic bacteria in humans can be beneficial for patients with familial adenomatous polyposis, the mice in our experiments did not confirm this observation. It might be due to different loca-

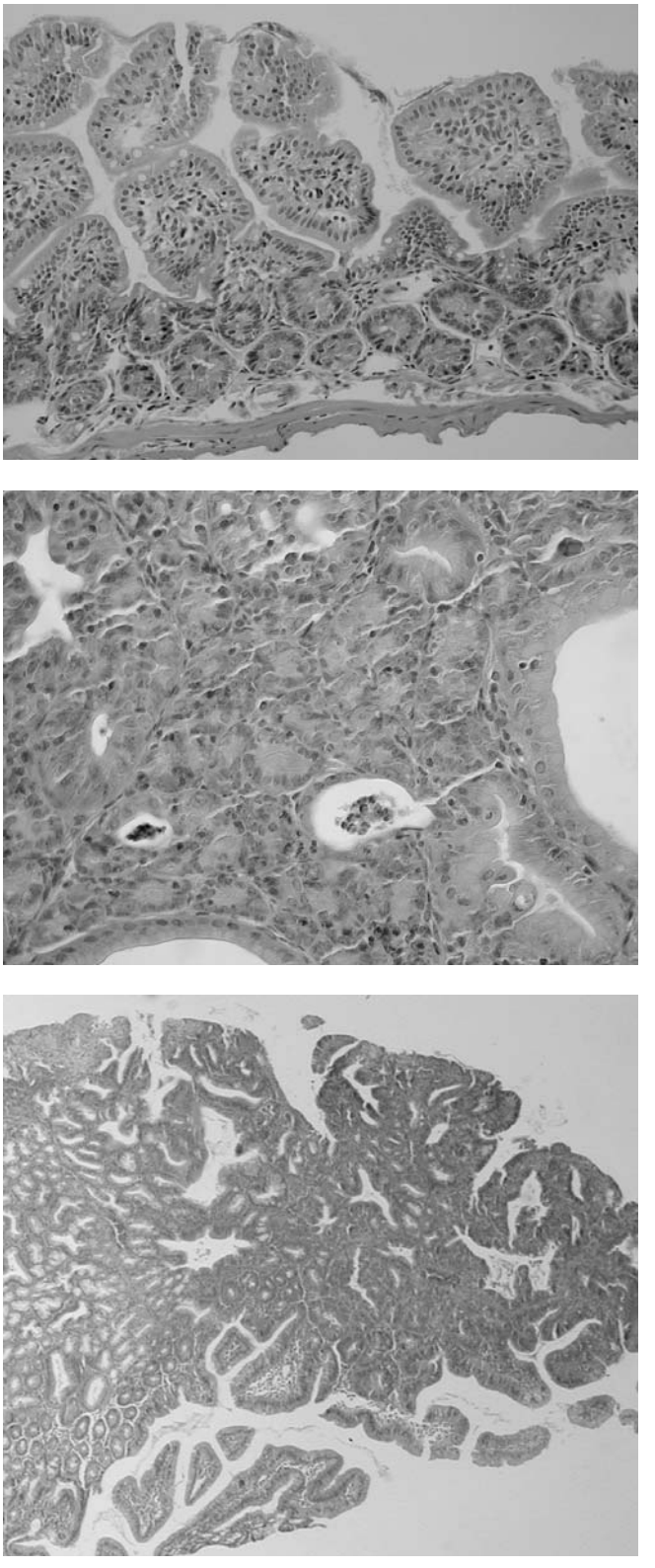

Figure 3. Examples of hematoxyline and eosine stained normal *Apc*$^{+}$/*Apc*$^{+}$ small intestine (A), (x100); small intestinal *Apc*$^{+}$/*Apc*^{1638N} tubular adenoma with moderate dysplasia, (B), (detail, x400) and tubulovillous adenoma with moderate dysplasia (C), (x40).

lization of adenomas in human and in mice and/or by different molecular mechanisms of tumor induction in mice versus humans. Clinical and animal model studies indicate that certain probiotic bacteria activate dendritic cells which have the potential of stimulating the mucosa-homing T lymphocytes within the Peyer's patches, to exert an immune outcome at distant mucosal sites [3].

Unlike the Foode's group where had developed the *Apc* transgenic mice [5], we did not observe progression to malignant adenomas in our experiments. It is possible that during the long-term maintenance of these animals natural selection for mice with better capability to survive occurred. This selection might be the reason for milder phenotype of the disease, without occurrence of adenocarcinomas and rectal bleeding, which was observed originally [5]. Our findings of 100% occurrence of tubular or tubulovilose adenomas with moderate dysplasia without occurrence of adenocarcinomas at the age of about 11 months was similar as was reported previously [10]. Although we did not observe rectal bleeding frequently, seven months old heterozygous males died only. We do not exclude the possibility that in some cases, when the dead animal was not suitable for authopsy the death may have been caused by progressively growing adenocarcinoma. The reduction of number of death in group of animals kept on enriched diet did not reflect any differences in histological findings.

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