Nontruncating APC Germ-line Mutations and Mismatch Repair Deficiency Play a Minor Role in APC Mutation-negative Polyposis

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ABSTRACT

Familial adenomatous polyposis, an autosomal-dominantly inherited colorectal cancer predisposition syndrome, is caused by germ-line mutations in the adenomatous polyposis coli (APC) gene. Despite the use of different screening methods, studies worldwide fail to identify APC mutations in 20–50% of all familial adenomatous polyposis patients (APC mutation-negatives). In this study, missense mutations in the coding region of the APC gene, which would have been missed by the protein truncation test, as well as mutations in the APC promoter and the 3′ untranslated region, were determined by the single nucleotide polymorphism discovery assay and direct DNA sequencing in 31 mutation-negative polyposis patients. Seventeen gene alterations were identified, whereas four (12.9%) represent possibly pathogenic germ-line mutations: silent A290T (promoter) and A882C (3′ untranslated region) as well as missense R99W and E1317Q (coding region). The 27 remaining, truly APC mutation-negative polyposis patients displayed a significantly later age at diagnosis compared with APC mutation carriers (46.1 versus 35.2 years; P < 0.01). APC mutation-negative individuals with >100 colonic polyps were more likely to present with extracolonic disease (P < 0.05) than those with <100. Assessment of microsatellite instability (MSI), a hallmark of mismatch repair deficiency, in 68 tumors from 21 truly APC mutation-negative patients, identified 4 (5.9%) unstable tubulo-villous adenomas (3 MSI-High and 1 MSI-Low), stemming from 4 (19%) unrelated individuals and likely to be caused by hMLH1 promoter hypermethylation. In conclusion, only a small proportion of APC germ-line mutation carriers is missed by the protein truncation test, and mismatch repair deficiency does not seem to substantially contribute to tumor development in APC mutation-negative polyposis patients.

INTRODUCTION

FAP5 (MIM 175100) is an autosomal dominantly inherited CRC predisposition syndrome. During adolescence, colonic polyposis ensues in virtually all FAP patients with the development of a few dozen to several thousands of adenomatous polyps throughout the colorectum, some of which eventually progress to CRC during the fourth and fifth decade if left untreated (1). According to the Danish Polyposis Register, the mean annual incidence amounts to 1.85 × 10−6 (time period, 1971–1992), with both sexes equally affected; FAP patients were found to account for approximately 0.1% of all Danish CRC patients (2).

FAP is caused by germ-line mutations within the APC gene, which was first localized to chromosome 5q21–22 in 1986 and finally cloned in 1991 (3–7). Applying different screening methods, like PTT and single strand conformation polymorphism analysis, more than 400 different germ-line mutations have been described, most of which lead to a truncated APC protein (8, 9). Despite these screening efforts, 20–50% of all classical FAP patients remain without a detectable APC germ-line mutation (so-called APC mutation-negative; Refs. 8, 10–15).

In addition, the disease displays considerable phenotypic heterogeneity. Even within so-called “typical” FAP families, polyp number and age of onset may vary considerably (reviewed in Ref. 16). The attenuated form of FAP (AAPC) has been characterized by a low (<100) and highly heterogeneous number of colonic polyps and later age of disease onset (17–19). AAPC is mainly observed with mutations located at the 5′ or the 3′ end of the APC gene, but may also be associated with missense variants, such as I1307K and E1317Q (20, 21).

In comparison with FAP patients with an identified mutation, APC mutation-negative polyposis patients display later age at diagnosis (45.3 versus 35.2 years) and 50% have <100 colorectal polyps (sometimes also referred to as multiple adenoma patients; Refs. 22, 23). Additionally, they tend to have fewer extracolonic manifestations (e.g., polyps of the upper gastrointestinal tract, desmoids, and osteomas). An extremely mild disease phenotype combined with a positive family history may mimic the clinical characteristics of another CRC predisposition, HNPCC. Intriguingly, recent observations suggest that some of these APC mutation-negative FAP families, unlinked to the APC locus, are linked to a new gene, CRCAI, on chromosome 15q (24). Other candidate genes for APC mutation-negative patients may include the MMR loci and the TGFBIRR locus (25).

To elucidate the molecular events in APC mutation-negative polyposis patients, the present study aimed to: (a) identify missense mutations in the coding region of the APC gene missed by the standard screening method (PTT); (b) assess the presence of mutations in the APC promoter and the 3′ UTR region; and (c) delineate the phenotypic characteristics of the truly APC mutation-negative polyposis patients; and (d) determine the extent to which MSI, a hallmark of MMR deficiency, contributes to the pathogenesis of APC mutation-negative polyposis.

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SUBJECTS AND METHODS

From March 1996 to August 2000, 100 unrelated Caucasian patients were referred by their treating physicians to the Department of Medical Genetics, University of Basel, Basel, Switzerland, for APC gene mutation analysis because of clinical diagnosis of polyposis coli. Written informed consent was obtained from all individuals.

In 31 (28%) polyposis coli patients, no truncating APC mutation in the germ-line could be detected (so-called APC mutation-negatives) using PTT, which has been described previously (26). Having no family history suggestive of HNPPC, all APC mutation-negative patients were analyzed additionally for the presence of germ-line mutations in the coding (GenBank accession no. M73548), the promoter (U02509), and the 3’ UTR region of the APC gene by applying two complementary methods, the SNP discovery assay (27) and DNA cycle sequencing. To assess the frequency of a given gene alteration in the population, a control panel of 146 healthy Caucasians was used, with 100 individuals stemming from the Coriell Cell Repositories (HD100CAU; Coriell Institute of Medical Research, Camden, NJ) and 46 healthy probands from the Medical Genetics Department, University Clinics, Basel, Switzerland.

DNA Extraction from Peripheral Blood. Genomic DNA was isolated from EDTA blood according to the method described previously (28). Briefly, 10 ml of blood were mixed with 30 ml of EL buffer (155 mm NH4 Cl, 10 mm KHCO3, and 1 ml EDTA (pH 7.4)) and left on ice for 15 min. The lysate was centrifuged at 2000 rpm for 10 min, washed twice with EL buffer, and then it was centrifuged to remove cellular proteins. The supernatant containing the DNA was placed in a fresh tube, and the DNA was precipitated with ethanol. The resulting DNA pellet was washed with 70% ethanol, dried briefly, and then suspended in 1 ml of TE buffer (10 mm Tris-HCl (pH 7.5) and 0.1 mm EDTA).

SNP Discovery Assay. Forty ng of genomic DNA were PCR-amplified in 50 ml of reactions using an MJ Research Tetrad PCR machine. To amplify the promoter region, coding sequence, and 3’UTR of the APC gene, primer pairs which amplify 300–600 bp fragments were designed either manually or using Primer3 software and GenBank sequence information (accession no. M73548). Standard reaction conditions included the following reagents: 10 mm Tris (pH 8.3), 50 mm KCl, 1.5 mm MgCl2, 0.2 mm of each dNTP, 0.4 pmol of each primer, and 1.5 units Taq Polymerase (Boehringer Mannheim, Mannheim, Germany). For some primer pairs the Invitrogen PCR buffer optimization kit was used for the adaptation of magnesium concentration and pH. For the amplification of GC-rich fragments, each 10% DMSO was added, or the amplification was done with solution Q from the Qiagen Hot Start Polymerase Kit. Qiagen Hot Start Taq Polymerase was used for a stringent PCR amplification.

The thermocycling protocol consisted of an initial incubation of 95°C for 15 min and then 35 cycles of 94°C for 1 min, 60°C for 30 s, 72°C for 1 min, and one final extension step of 72°C for 10 min. After PCR amplification, fragments were purified with the Qiagen Quick PCR purification kit on a Biorobot 9600. Cycle sequencing was performed on an MJ Research Tetrad PCR machine using ABI Big Dye Terminator chemistry according to the manufacturer’s instructions, with the following changes: 2–4 ml of purified PCR product was mixed with 2 ml of Big Dye Terminator Ready-Reaction Promix and 10 pmol of sequencing primer; 5% DMSO for GC-rich sequences. The final reaction volume was 10 ml. Reactions were subjected to 28 cycles at 93°C for 30 s, 48°C for 30 s, and 58°C for 2 min and then an ethanol precipitation. After decanting the ethanol, samples were evaporated to dryness using a SpeedVac for 2 min and resuspended in 45 ml of ultrapure water. An ABI 3700 capillary sequencer was loaded with 2.5 ml using POP5 as a polymer.

SNP Analysis. The sequencer-generated data were transferred to a Sun server, where the analysis software, a suite of sequence assembly tools (Phred, Phrap, Polypyred, and Consed), resides (29–31). Phred is the base caller, which also assigns a quality score to each position in the read (29, 30). Phrap assembles the reads into a contig. Consed is the analysis viewer (31), a graphical user interface to view or edit the assembly created by Phrap. Polypyred has an algorithm to detect heterozygote polymorphisms in an assembly (32). Because reads from PCR products may, in some cases, continue beyond the physical end of the DNA, the low quality sections of the reads were removed from the end of the read files before Phrap and Polypyred analysis. Phrap and Polypyred generated “tags,” which were used to indicate the position and nature of different sequence features. Whereas Phrap-generated tags essentially deal with features relevant to the assembly of the contig, Polypyred-generated tags indicate the position and type of observed polymorphisms. These results were verified manually.

Cycle Sequencing. Cycle sequencing was performed using the Thermo Sequenase cycle sequencing kit according to the manufacturer’s instructions (Amersham Pharmacia, Dübendorf, Switzerland). The primers were end-labeled with 32P IR dye, IRD-800 (MWG Biotec, Ebersberg, Germany), and used directly in the cycle sequencing reaction. Briefly, 2–5 ml of PCR product were diluted to a final volume of 7 ml. Ten units of exonuclease I and 2 units of shrimp alkaline phosphatase (both Amersham Pharmacia) were added to remove excess primers and dNTPs (at 37°C for 15 min and 80°C for 15 min). To each sample, dH2O was added to obtain a final volume of 25 ml from which 6 ml were combined with 2 ml of A, C, G, or T reagent, respectively. Cycle sequencing conditions were 95°C for 4 min, as an initial denaturating step, and then 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Finally, 3 ml of red stop dye were added to accomplish the reaction. After denaturation at 95°C for 5 min, 1 ml of the reaction mix was loaded on a 6% denaturing polyacrylamide gel and analyzed on an automated DNA sequencer according to the manufacturer’s instructions (LI-COR, Lincoln, NE).

Tumor DNA Extraction. After histopathological classification of H&E-stained tissue slides, a representative portion of the tumor (adenoma or carcinoma) with an average tumor content of ≥70% was scraped off, and DNA extraction was performed according to the QIAamp kit protocol (Qiagen, Basel, Switzerland). Briefly, 180 ml of buffer ATL and 20 ml protease K (20 ng/ml) were added to each tumor sample, which then were incubated overnight at 55°C for digestion until the tissue was completely lysed. Subsequently, 200 ml of buffer AL were added and incubated at 70°C for 10 min, with the subsequent addition of 210 ml of ethanol (100%), and mixed thoroughly by vortexing. Applied on a QIAamp spin column, the lysate was centrifuged at 10,000 rpm for 3 min. After having discarded the filtrate, 500 ml of washing buffer AW were added twice and centrifuged at full speed (14,000 rpm). Finally, the DNA was eluted twice with 200 ml of buffer AE.

Microsatellite Marker Analysis. Ten microsatellite markers were chosen based on recommendations of the National Cancer Institute workshop on MSI. The first panel included the microsatellite loci BAT25, BAT26, D5S346, D17S250, and D2S123, which were analyzed using a commercially available kit (Roche Diagnostics, Basel, Switzerland). The second panel of microsatellites included BAT40, MYCLI, D10S197, D18S58, and D18S569, which were optimized for multiplex PCR. One hundred ng of leukocyte DNA and 200 ng of tumor DNA were used to perform the PCR under the following conditions: first panel: initial denaturation at 94°C for 2 min and then 30 cycles at 94°C for 10 s, 55°C for 30 s, and 72°C for 30 s with a final extension step at 72°C for 7 min; second panel: initial denaturation at 94°C for 3 min and then 30 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min. Two ml of PCR product were added to 10 ml formamide (including 0.5 ml GS size standard 350 Tamra), denatured at 95°C for 5 min, chilled on ice, and loaded on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Rotkreuz, Switzerland). The interpretation of the presence of MSI, defined as the occurrence of novel alleles, followed the recommendations of the National Cancer Institute workshop: none of the microsatellite markers unstable, stable (MSS); >0–≤30% of markers unstable, low instability (MSI-L); >30% of markers unstable, high instability (MSI-H). Tumor samples from HNPPC patients were included as positive controls. Microsatellite marker analysis also allowed the identification of LOH, commonly defined as a ≥50% reduction in relative intensity of one allele compared with the other (33).

Immunohistochemical Analysis. Four-μm serial sections from paraffin blocks were mounted on siliconized slides, deparaffinized, and rehydrated. Antigen retrieval was obtained by heating the sections in a pressure cooker at 120°C for 2 min in 10 mm citrated-buffered solution (pH 6.0). DAKO peroxidase blocking reagent and goat serum were sequentially used to suppress nonspecific staining caused by endogenous peroxidase activity and nonspecific

binding of antibodies, respectively. Incubations with primary monoclonal antibodies were performed as follows: anti-hMSH2, 24 h at 4°C with Ab NA26 (Oncogene Research), 1 μg/ml; anti-hMSH6, 2 h at room temperature with Ab G70220 (Transduction Laboratories), 4 μg/ml; anti-hMLH1, 1 h at room temperature with Ab 13271A (PharMingen), 1.2 μg/ml; and anti-hPMS2, 24 h at 4°C with Ab 65861A (PharMingen), 3 μg/ml. After washing, antimouse secondary antibodies conjugated to peroxidase-labeled polymer (DAKO EnVision +kit) were applied for 30 min at room temperature, and the peroxidase activity was developed by incubation with 3,3′-diaminobenzidine chromogen solution (DAKO). Sections were then counterstained slightly with hematoxylin.

Despite the use of several commercially available antibodies against APC, non reproducible and reliable immunohistological staining patterns could be established to directly assess the loss of APC protein expression in the tumors available.

**Phenotypical Characteristics and Statistical Analysis.** The APC mutation-negative polyposis patients were compared using phenotypical data (gender, age at diagnosis, polypos number, CRC occurrence, and extracolonic disease) and molecular genetic data (mutational analysis, MSI, and LOH). The term “multiple,” noted in the medical records of some patients, describes adenoma numbers between >5 and <100 adenomas (34). For statistical analysis, the χ², Fisher’s exact, and Student t tests were used when appropriate, with all probabilities reported as 2-tailed P, considering a P ≤ 0.05 to be significant (Excel 2000; Microsoft Corp.).

**RESULTS**

From a consecutive series of 100 patients clinically diagnosed as having polyposis coli between March 1996 and August 2000, 31 (31%) were found to carry no detectable truncating APC mutation as assessed by PTT. These so-called APC mutation-negative polyposis patients were consecutively screened for germ-line mutations within the coding region of the APC gene as well as the promoter and the 3’ UTR. Furthermore, interindividual phenotypic differences and the presence of MSI in colonic tumors (mostly adenomatous polyps) from the truly APC mutation-negative patients were assessed. Because of the unavailability of additional family members and/or the uninformativeness of the markers used, linkage to the APC locus could not be established in APC mutation-negative patients with a positive family history.

**Mutational Analysis of the Promoter, Coding and 3’ UTRs of the APC Gene.** Applying the SNP discovery assay and direct DNA sequencing on 31 APC mutation-negative polyposis patients, 17 germ-line variants were detected within the APC promoter, coding, and 3’ UTR (Table 1). Thirteen variants were deemed to be nonpathogenic, because they did not lead to an amino acid change and thus did not have an obvious consequence on the protein level. Although possible effects on transcription and splicing efficiency cannot be excluded (35), these polymorphisms were also present in healthy control persons and did not cosegregate with the disease phenotype within the family (Table 1). Therefore, they have been considered as nonpathogenic polymorphisms, and the patients carrying them have been considered as truly APC mutation-negative.

In four (12.9%) patients, four different, possibly pathogenic, mutations were identified: whereas the missense mutations R99W and E1317Q have been reported previously (20, 36), the A290T mutation, located within the APC promoter, as well as the A882G mutation in the 3’ UTR of the APC gene represent novel mutations and are, to our knowledge, the first mutations described within regulatory regions of APC.

The R99W missense mutation (C358T) was identified in a 64-year-old woman (1506) with a positive family history of CRC and diagnosed of having <100 adenomas, predominantly located in the ascending colon and of tubular origin, and having no extracolonic disease at presentation. The missense mutation was identified also in her daughter, who did not develop polyps until 38 years of age. Male patient 1762, harboring the E1317Q mutation (G4012C), without a family history of polyposis and/or CRC, was diagnosed as having about 90 mostly tubular and tubulo-villous adenomas in the large intestine as well as moderately differentiated adenocarcinoma of the rectum at 44 years of age. No extracolonic disease was reported.

In the family of the male patient 1551, who was diagnosed as having <100 colorectal adenomas at 47 years of age, the A290T

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**Table 1**  **Point mutations identified in a consecutive series of apparently APC mutation-negative polyposis coli patients (n = 31)**

<table>
<thead>
<tr>
<th>Region</th>
<th>DNA change</th>
<th>Mutation consequence</th>
<th>Cosegregation</th>
<th>Frequency in control population</th>
<th>Interpretation</th>
<th>Patient no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>A209T</td>
<td>Unknown</td>
<td>Yes</td>
<td>0%</td>
<td>Possibly pathogenic</td>
<td>1551</td>
</tr>
<tr>
<td>Exon 1B</td>
<td>A161C</td>
<td>Silent</td>
<td>ND</td>
<td>5 (10.9%)</td>
<td>Polymorphism</td>
<td>1666, 1743</td>
</tr>
<tr>
<td>Exon 1B</td>
<td>C254T</td>
<td>Silent</td>
<td>ND</td>
<td>6 (13%)</td>
<td>Polymorphism</td>
<td>1736</td>
</tr>
<tr>
<td>Exon 1B</td>
<td>274delG</td>
<td>Unknown</td>
<td>No</td>
<td>1 (2.2%)</td>
<td>Polymorphism</td>
<td>1749</td>
</tr>
<tr>
<td>Intron 5</td>
<td>C9644T</td>
<td>Unknown</td>
<td>ND</td>
<td>24 (52.2%)</td>
<td>Polymorphism</td>
<td>1552, 1665, 1719, 1763, 1767, 1828</td>
</tr>
<tr>
<td>Exon 3</td>
<td>C358T</td>
<td>R99W</td>
<td>Yes</td>
<td>ND</td>
<td>Pathogenic</td>
<td>1506</td>
</tr>
<tr>
<td>Exon 15</td>
<td>A3203T</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1699</td>
</tr>
<tr>
<td>Exon 15</td>
<td>G4012C</td>
<td>E1317Q</td>
<td>ND</td>
<td>ND</td>
<td>Possibly pathogenic</td>
<td>1762</td>
</tr>
<tr>
<td>Exon 15</td>
<td>T4367A</td>
<td>Silent</td>
<td>ND</td>
<td>4 (8.7%)</td>
<td>Polymorphism</td>
<td>1764, 1803</td>
</tr>
<tr>
<td>Exon 15</td>
<td>A6568G</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1842</td>
</tr>
<tr>
<td>Exon 15</td>
<td>C7242T</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1545</td>
</tr>
<tr>
<td>Exon 15</td>
<td>A4520G</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1545, 1719, 1821, 1522, 1717, 1762, 1812, 107, 1699, 1803, 18, 1505, 1588, 1777, 1719, 14, 1645, 1775, 10, 1778, 1459, 1552, 1771, 1859, 1384, 1640, 1767, 1842, 1097, 1551, 1736, 1764</td>
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<tr>
<td>Exon 15</td>
<td>A5075G</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1545, 1719, 1821, 1522, 1717, 1762, 1812, 107, 1699, 1803, 18, 1505, 1588, 1777, 14, 1645, 1775, 10, 1778, 1459, 1552, 1771, 1859, 1384, 1640, 1767, 1842, 1097, 1551, 1736, 1764</td>
</tr>
<tr>
<td>Exon 15</td>
<td>G5309T</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1545, 1719, 1821, 1522, 1717, 1762, 1812, 107, 1699, 1803, 18, 1505, 1588, 1777, 14, 1645, 1775, 10, 1778, 1459, 1552, 1771, 1859, 1384, 1640, 1767, 1842, 1097, 1551, 1736, 1764</td>
</tr>
<tr>
<td>Exon 15</td>
<td>A5921G</td>
<td>Silent</td>
<td>ND</td>
<td>ND</td>
<td>Polymorphism</td>
<td>1545, 1719, 1821, 1522, 1717, 1762, 1812, 107, 1699, 1803, 18, 1758, 1777, 14, 1645, 1775, 10, 1778, 1459, 1552, 1771, 1859, 1384, 1640, 1767, 1842, 1097, 1551, 1736, 1764</td>
</tr>
</tbody>
</table>

- a Control population consisted of 146 healthy Caucasian individuals.
- b ND, not determined.
- c Control population consisted of 46 healthy Caucasian individuals.
- d One caucasian control was homozygous for the mutation.
germ-line mutation was found to cosegregate with disease (i.e., polyposis and/or CRC) in two affected family members, whereas an unaffected family member (age 41) was found to carry only wild-type alleles of the gene. The A290T promoter mutation was absent in a control population consisting of 146 healthy Caucasian individuals.

The 3' UTR A8822G germ-line mutation was identified in a male patient (1736), who was diagnosed at 46 years of age as having classical colonic polyposis (>100 adenomas) as well as 2 synchronous adenocarcinomas of the caecum and descending colon, respectively. Because of the negative family history, no cosegregation analysis could be performed. In the Caucasian control population, however, the A8822G mutation was found in only one (0.7%) individual.

Subsequently, the four individuals carrying possibly pathogenic APC germ-line mutations were excluded from the additional phenotypic analysis of truly APC mutation-negative polyposis patients.

**Phenotypic Properties of Truly APC Mutation-negative Patients.** The phenotypic properties of the 27 APC mutation-negative polyposis patients are depicted in Table 2. The sex ratio (male:female) was 16:11 (59%:41%) and did not significantly covary with other phenotypic characteristics (i.e., age, family history, polyp number, presence of CRC and extracolonic disease). The polyposis was clinically diagnosed at a mean age of 46.1 years (±14.7, SD; range, 20–73), which is significantly later compared with our APC-positive patients’ mean age (35.2 years ± 13.7, SD; P < 0.01). Although not statistically significant (P = 0.20), women were found to be diagnosed on average 7.8 years earlier than men (41.5 ± 17.2 years versus 49.3 ± 12.3 years, respectively). In addition, there were no significant differences in age distribution when grouped according to family history, polyp number, occurrence of CRC, and extracolonic manifestations. Subdividing the APC mutation-negative polyposis patients according to the polyp number observed at diagnosis (<100/multiple versus >100 adenomas) demonstrated that patients exhibiting >100 adenomas were more likely to present with extracolonic disease (Fisher’s exact test; P < 0.05). They did not, however, differ with respect to any other phenotypic characteristics investigated; in particular, a similar percentage of patients with a positive and those with a negative family history displayed >100 polyps at diagnosis (25 and 27%, respectively). Extracolonic disease tended to be more often observed in patients with a positive family history (71%; n = 7) compared with those without (35%; n = 20), although this was not statistically significant (P = 0.1).

**MSI in Tumors from APC Mutation-negative Polyposis Patients.** From 21 (78%) of 27 APC mutation-negative individuals, paraffin-embedded adenoma and/or adenocarcinoma tissue specimens were available for MSI testing. Importantly, the 21 patients displayed nearly identical phenotypic properties (i.e., gender, age at diagnosis, family history, polyp number, CRC and extracolonic disease) when compared with the overall group (data not shown). In total, 77 tissue specimens encompassing 74 colorectal adenomas and 3 adenocarcinomas were analyzed, with MSI status established in 68 (88%) samples. In addition, tumor tissue (n = 13) from all four patients (1506, 1551, 1736, and 1762) harboring possibly pathogenic APC germ-line mutations as well as adenocarcinomas from two hMLH1 mutation carriers (proven MSI-H) were investigated as controls.

The results of the MSI analysis for each of the 21 APC mutation-negative polyposis patients are summarized in Table 3. Four (5.9%) of 68 tumors were found to exhibit a low (MSI-L) or high (MSI-H) degree of instability, almost exclusively in dinucleotide repeats. Histologically, they were characterized as moderately differentiated, tubular, or tubulo-villous adenomas, stemming from four (19%) unrelated individuals. Significantly, all unstable adenomas belonged to individuals having >100 colonic adenomas at diagnosis (P < 0.01), with two patients also having a positive family history. All adenomas (n = 13) coming from the four patients with possibly pathogenic APC germ-line mutations were found to be stable (MSS), and the MSI-H status of positive controls could be confirmed.

From patient 1465, two tubulo-villous adenomas from the descending and the sigmoid colon could be investigated, with one being MSI-H [four of eight markers (50%)] and one MSS for all markers investigated. Unfortunately, immunohistochemical analysis of the unstable adenoma was inconclusive, because the biopsy specimen was too small and did not include normal mucosal epithelium for adequate comparison. The woman was 64 years of age when colonic polyposis

### Table 2: Phenotypic properties of 27 APC mutation-negative polyposis patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Family history</th>
<th>Age at diagnosis (yr)</th>
<th>No. of polyps</th>
<th>CRC</th>
<th>Age at diagnosis (yr)</th>
<th>CRC site</th>
<th>Extracolonic disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1384*</td>
<td>F</td>
<td>Positive</td>
<td>20</td>
<td>Multiple</td>
<td>No</td>
<td>67</td>
<td>Ascending</td>
<td>Duodenal polyposis</td>
</tr>
<tr>
<td>1459</td>
<td>M</td>
<td>Positive</td>
<td>66</td>
<td>Multiple</td>
<td>Yes</td>
<td>53</td>
<td>Rectum</td>
<td>No</td>
</tr>
<tr>
<td>1465</td>
<td>F</td>
<td>Positive</td>
<td>64</td>
<td>&gt;100</td>
<td>No</td>
<td>73</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>1505</td>
<td>F</td>
<td>Negative</td>
<td>45</td>
<td>&gt;100</td>
<td>Yes</td>
<td>53</td>
<td>Rectum</td>
<td>No</td>
</tr>
<tr>
<td>1522*</td>
<td>M</td>
<td>Negative</td>
<td>46</td>
<td>Multiple</td>
<td>Yes</td>
<td>46</td>
<td>Rectum</td>
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</tr>
<tr>
<td>1545</td>
<td>M</td>
<td>Negative</td>
<td>54</td>
<td>&lt;100</td>
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* = individuals from which no tumor tissue was available for assessing microsatellite instability.
(>100 adenomas) was discovered, with no apparent CRC or extracolonic disease present. In two family members (her son and her mother) polyposis coli had been diagnosed at 31 and 73 years of age, respectively, with the latter individual having also developed cancer of the ascending colon.

From patient 1505, two tubulo-villous adenomas from the distal colon as well as a rectal adenocarcinoma were available for study, with one adenoma displaying MSI-H [three of eight markers (38%)] and the remaining two tissues being MSS. Immunohistochemical analysis (for hMLH1, hMSH2, hMSH6, and hPMS2) of the unstable adenoma revealed a very weak hMLH1 staining pattern in the adenoma portion when compared with adjacent normal mucosa. The woman was diagnosed with polyposis (>100 adenomas) at 45 years of age, when rectal cancer had been discovered also. Her family history was negative for polyposis and/or CRC, and no extracolonic disease was present.

For patient 1777, a total of nine tumor specimens were investigated, encompassing four tubular and four tubulo-villous adenomas as well as an adenocarcinoma of the rectum. One tubular adenoma was found to display a low degree instability at marker D18S58 [one of eight markers (13%)] In contrast to hMSH2, hMSH6, and hPMS2, the hMLH1 protein was found to display a markedly milder phenotype (>100 adenomas) at 22 years of age, without a family history of polyposis/CRC or extracolonic disease. Twenty-two years after subtotal colectomy, she developed a rectal adenocarcinoma at 44 years of age.

For patient 1552, one of four tubulo-villous adenomas investigated was found to display a low degree instability at marker D18S58 [one of eight markers (13%)] In contrast to hMSH2, hMSH6, and hPMS2 proteins, being equally present in adenoma and normal mucosa, the hMLH1 protein staining pattern was very weak in the adenoma portion. The man had a negative family history of CRC and was diagnosed with polyposis coli (>100 adenomas) at 72 years of age, with no CRC present. At 76 years of age, he developed an abdominal desmoid tumor at the site where the colectomy had been performed 4 years before.

LOH was observed in various adenomas from different APC mutation-negative individuals, predominantly involving the dinucleotide repeat markers D18S58 and D18S69, both located on chromosome 18q21 close to the tumor suppressor loci DCC and SMAD4, as well as MYCL1, a tetranucleotide repeat, located upstream of L-myc on chromosome 1p34 (Table 3).

**DISCUSSION**

In this study, 31 APC mutation-negative patients, stemming from a consecutive series of 100 polyposis coli patients and carrying no truncating APC germ-line mutation as determined by PTT, underwent extensive APC mutational screening using the SNP discovery assay and direct DNA sequencing. Four (13%) possibly pathogenic germ-line mutations were identified in the coding sequence (R99W and E1317Q) as well as in the promoter (A290T) and the 3’ UTR (A8822G) of the gene.

In agreement with previous reports on genotype/phenotype correlations at the 5’ end of the APC gene, the patient harboring the R99W mutation displayed a markedly milder phenotype (i.e., <100 polyps, later age at diagnosis, absence of extracolonic disease; Refs. 17, 37). Although a matter of ongoing debate, the E1317Q missense mutation is believed either to directly exert a (mildly?) pathogenic effect by a yet-undetermined mechanism or to hallmark a cryptic APC mutation nearby, predisposing to the development of multiple colorectal adenomas and carcinoma with a variable degree of penetrance (20). In agreement with previous reports, the patient displayed <100 polyps at the time of diagnosis of rectal cancer at 44 years of age (20).

The A290T promoter mutation was found to cosegregate with disease and was absent in a control series of 146 Caucasian controls, which clearly argues in favor of the mutation being pathogenic. Thus far, no firm conclusions can be made concerning the pathogenicity of the A8822G germ-line alteration in the 3’ UTR of the APC gene, because cosegregation analysis was not possible, and 1 of 148 healthy Caucasian controls displayed the same mutation. Clearly, systematic screening of the 3’ UTR region in other APC mutation-negative collectives as well as investigations on the functional relevance of the A8822G mutation are needed to determine its putatively pathogenic role. Moreover, use of the diploid-to-haploid gene conversion method should help to evaluate the significance of the mutations within the regulatory regions and definitely exclude the presence of masked APC mutations (38).

Albeit with limitations (i.e., failure to detect large genomic deletions/insertions), the results of the current extensive mutation analysis indicate that only a small proportion (4 of 31; 12.9%) of APC mutation carriers is missed by standard truncation detection techniques such as PTT, and that in a considerable percentage of polyposis patients (27 of 100; 27%) other, yet-undetermined, pathogenetic mechanisms may play a role. In particular, previous work failed to...
identify pathogenic germ-line mutations in another member of the Wnt signaling pathway, the β-catenin gene, in 6 of 27 APC mutation-negative patients of the current survey (39). It remains to be seen, however, to which degree mutations in other members of the Wnt pathway, such as axin1 and axin2, contribute to the disease.

The age at diagnosis of the remaining 27 truly APC mutation-negative polyposis patients significantly differed from mutation-positive individuals, in that the former were diagnosed 10 years later on average (46.1 versus 35.2 years; P < 0.01). However, despite the significant difference in age at diagnosis, both groups were diagnosed with CRC 20–30 years (fourth and fifth decades) earlier than the general Swiss population (seventh decade; Swiss Cancer Registries’ Association database, 1996), pointing toward an inherited genetic disorder rather than a sporadic event. Overall, the phenotypic properties such as age at diagnosis, number of polyps, polypt site, and histology, as well as development of CRC and extracolonic disease, did not significantly differ among the APC mutation-negative polyposis patients, with one exception: patients exhibiting >100 adenomas presented more frequently with extracolonic disease than patients with multiple/≤100 adenomas (P < 0.05). Finally, as our study population represents a highly selected group, i.e., APC mutation-negative polyposis patients, an ascertainment bias cannot be ruled out.

To determine the degree to which a deficient MMR system might contribute to the pathogenesis in APC mutation-negative patients, MSI analysis was assessed in 68 adenomas from 21 patients, identifying MSI in 4 (5.9%) adenomas (3 MSI-H and 1 MSI-L) from 4 (19%) different patients. Significantly, all four patients had developed >100 adenomas at diagnosis. The proportion of unstable adenomas was comparable with other reports on MSI in FAP and sporadic polyposis (3%; Ref. 40). Immunohistochemically, hMLH1 staining was very weak in two of three adenomas suitable for analysis, both stemming from patients with a negative family history. As MSI did not occur in all tumors analyzed from the same patient, these findings point to the somatic inactivation of hMLH1 by promoter hypermethylation as frequently observed in unstable sporadic adenomas and CRCs, rather than to the presence of an hMLH1 germ-line alteration (41, 42). Taken together, MSI in adenomas from APC mutation-negative polyposis patients seems to be a rare event, comparable with frequencies observed in sporadic ones, and mainly attributable to hMLH1 inactivation, most likely by promoter hypermethylation. Thus, MMR deficiency is unlikely to account for tumor development in APC mutation-negative polyposis patients, which stands in agreement with previous reports assessing the frequency of MMR gene mutations in APC mutation-negative multiple adenoma patients (43). Moreover, a study on familial CRC patients without polyposis coli and reminiscent of HNPCC found no pathogenic germ-line alterations in the 5′ APC region (i.e., exons 1–6) of the APC gene, indicating that mutations in this region are infrequent in familial nonpolyposis CRC patients (44).

In conclusion, our findings indicate that only a small proportion (13%) of APC germ-line mutation carriers is missed by conventional screening techniques (i.e., PTT), and that in 30% of polyposis patients no alterations within the APC gene can be identified. These APC mutation-negative polyposis patients are characterized by a significantly later age at diagnosis, compared with mutation carriers, and patients with >100 polyps are more frequently found to present with extracolonic disease. As MSI was present in only a fraction of adenomas from APC mutation-negative polyposis patients, a significant contribution of MMR deficiency to the tumorigenesis in these patients seems unlikely. Future research on the molecular genetic events and pathways involved in tumor development in APC mutation-negative polyposis patients may not only help to improve genetic counseling and medical care for these patients and their families, but may also shed additional light on the genetic basis of CRC in general.

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Nontruncating \textit{APC} Germ-line Mutations and Mismatch Repair Deficiency Play a Minor Role in \textit{APC} Mutation-negative Polyposis

Karl Heinimann, Annick Thompson, Andreas Locher, et al.


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